

**The Role of Highly Phosphorylated Nucleotides in the  
Development of  
Bacterial Resistance to Antibiotics**

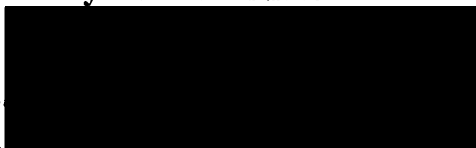
**Jane Leitch, BSc(Hons)**

**A Thesis submitted in partial fulfilment of the requirements of the  
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for the degree of  
Doctor of Philosophy**

**June 1998**

**I certify that this thesis is the true and accurate version of the thesis  
approved by the examiners.**

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## ABSTRACT

The term 'Magic Spot' (MS) defines a small group of highly phosphorylated purines (HPNs; MSI, ppGpp; MSII, pppGpp) produced by bacterial cells during the late exponential and early stationary phases of growth, in response to adverse nutritional conditions. These compounds are involved in the phenomena of endospore formation and stringent response which both elicit an increase in observed antibiotic resistance in many bacterial species. It is thought that HPNs indicate the onset of and/or initiate bacterial resistance to the presence of antibiotics. The effects of nutrient limitation (carbon, nitrogen, magnesium and phosphate) upon the two bacterial species, *Escherichia coli* NCIMB 10000 and *Bacillus subtilis* NCIMB 12900, were observed. The appearance and disappearance of various nucleotides (AMP, GMP, ADP, GDP, ATP and GTP) involved in HPN biosynthesis in *E. coli* and *B. subtilis* grown in nutrient-limited media were observed *via* fluorescence 2D-TLC. Cellular extracts of both *E. coli* and *B. subtilis* exhibited a reduction in the apparent content of ATP, GTP, ADP and GDP when observed by 2D-TLC. A fluorescent spot was observed at the origin which was assumed to correspond to 'Magic Spot'. Results indicate that ATP is the pyrophosphate donor in HPN synthesis and that GDP and GTP are the acceptor molecules. A new rapid isocratic HPLC protocol was developed in this study, in order to quantify the 2D-TLC results. Cellular extracts taken from batch cultures of *E. coli* and *B. subtilis* grown under conditions of nutrient limitation, both challenged and unchallenged with sub-minimal inhibitory concentrations (MIC) of erythromycin, exhibited a reduction in the concentration of AMP, GMP, ADP, GDP, ATP and GTP with the concurrent synthesis of HPNs. The HPNs exhibited peaks with  $R_t$  values of 3.8 mins (ppGpp, MSI) and 2.5 mins (pppGpp, MSII) respectively. Further experiments involving the fluorimetric analysis of ATP concentration in growing bacterial cultures revealed a marked decrease in ATP concentration per cell at the onset of stationary phase and support the previous observations regarding the role of this compound in the synthesis of HPNs. The new isocratic HPLC protocol was utilised for the observation of the kinetics of nucleotide and HPN concentrations in cellular extracts taken from batch cultures of both microorganisms. These results suggest a close relationship between the reduction and disappearance of certain nucleotides and the synthesis and appearance of HPNs. The detection of HPNs in the cultures challenged with sub-MIC erythromycin, at either the onset of or during stationary phase of growth, indicates that the onset of sporulation and/or stringent response is closely associated with the onset of bacterial resistance to this antibiotic.

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## INTRODUCTION

### Natural Growth

In a natural, constantly changing environment, bacteria exist in a dynamic state adapting both genotypically and phenotypically in response to these changes and thus, supporting balanced growth and survival (Roszak & Colwell 1987, Chesbro 1988). The nutrient availability in natural ecosystems tends to be scarce, with low levels of carbon and insoluble phosphorous and iron (Matin *et al* 1989). The consequences of this nutrient scarcity for the natural microflora are sub-maximal or zero growth rates. When unfavourable environmental changes occur, the microbial population reduces its growth rate. This rate often approaches zero, however, the bacteria remain viable (Brown *et al* 1990). The growth rate of bacteria in the natural environment is dependent upon the bacteria's ability to modify its metabolism in order to maintain and survive in the extreme conditions encountered. The conditions in which the bacteria grow are normally depleted of essential requirements, namely oxygen and nutrients. These problematic, but normal conditions must be considered if a holistic approach is to be achieved when attempting to mimic bacteria's natural growth regimes in the laboratory.

Bacteria grow and divide solely in order to survive. In the natural environment limitations of substrate produce alternate patterns of growth and non-growth. In marine ecosystems bacterial populations are subjected to a broad range of discontinuities, such as interfaces where the nutritional concentrations and substrate compositions are widely varied (Kjelleberg *et al* 1987). The fluctuations encountered by the bacteria can induce an intermittent mode of growth, including periods of nongrowth during which the bacteria possess the ability to capture substrates at concentrations which maintain viability, but does not sustain an increase in biomass (Kjelleberg *et al* 1987).

Carbon is available for microbial growth as either carbon dioxide or organic matter. The concentrations of organic carbon in most environments may be substantially higher than the concentrations of inorganic carbon matter, but due to the variety of organic compounds, each of which is transported individually into the cell, there may well be insufficient concentrations of any single compound to saturate the appropriate uptake systems (Tempest *et al* 1983). Natural aquatic environments generally contain only low

concentrations of readily assimilated nitrogen or phosphorous. More specific environments may be depleted of either magnesium, potassium and/or phosphorous. It is reasonable to assume that microorganisms will frequently experience basic nutrient limitation. The microorganisms in these environments are highly adept at coping with these growth limiting conditions. There are four potential modifications in bacterial behaviour in environments of nutrient depletion (Tempest *et al* 1983):

- (1) synthesis of enzymes specific for the improvement of utilisation of available nutrients
- (2) modulation of the rate of uptake and metabolism of rate limiting nutrients
- (3) rearrangement of their metabolism to evade bottlenecks imposed by the specific growth limitation
- (4) modulation of the rate of synthesis of macromolecular components to permit balanced growth.

Monod (1942) proposed that the relationship between growth rate and substrate concentration followed Michaelis-Menten kinetics, where some component of the system becomes saturated (limiting) at low substrate concentrations. Growth is said to be balanced when all the components of the biomass change by the same constant factor per time unit. During growth the substrate is depleted and the specific growth rate constant  $\mu$ , becomes dependent on the growth limiting substrate concentration,  $c$  (Neidhardt *et al* 1990). The non-growth phase of bacteria can be determined by measuring the productivity and specific growth rates  $\mu$ . Monod (1942), by reference to Michaelis-Menten (1913) formulated the specific growth rate in relation to the substrate concentration as follows (Equation 1).

$$(1) \quad \mu = \mu_{\max} c / (K_s + c)$$

Where  $\mu$  is the specific growth rate at a concentration,  $c$  of the limiting substrate and  $K_s$  is a constant that is numerically equal to the substrate concentration at which  $\mu = \mu_{\max}/2$ . Monod's equation simplifies growth to a process in which the total metabolic rate is determined by the substrate uptake rate (Arbige *et al* 1993). This approach treats the microorganism as a collection of enzymes which responds to nutrient limitation as would a single enzyme. Monod (1949) also suggested that the exponential growth rate was either reduced or halted by any one, or combination of the following four factors (i) build up of

toxic end products, (ii) unfavourable pH attained during growth, (iii) ionic imbalance and (iv) nutrient limitation. Both growth rate and yield is dependent upon the substrate which serves as the carbon source. Schaechter *et al* (1958) supported this theory when they observed a systematic change in size and composition of *Salmonella typhimurium* when growth rate varied due to nutrient limitation. Bacterial productivity is the rate of increase of biomass and is directly proportional to nutrient availability (Schaechter *et al* 1958). The specific growth rate  $\mu$  is the number of generations occurring per unit of time (cell production rate divided by the number of cells present) (Marr 1991).

The fluctuations in nutrients available to the bacteria result in changes in specific growth rates (Kjellberg *et al* 1993). These fluctuations in natural environments occur over time scales varying from hours to seasons. Thus, the bacteria can experience varied time periods of stress. In order to ensure bacterial survival, their metabolism is adapted to cope with these conditions. Bacteria respond to starvation in many ways, these include dormancy, stringent response in non-sporulating bacteria and the formation of endospores in sporeforming bacteria.

### *Biofilms*

In nature planktonic populations of microorganisms rarely exist. The large majority of viable bacteria tend to be found on surfaces in the form of biofilms. Biofilms are naturally occurring colonies of species of diverse microorganisms which grow adherent to natural and synthetic surfaces (Costerton 1984, Costerton & Lashen 1984, Costerton *et al* 1988, Geesey & White 1990, Gilbert *et al* 1993, Costerton 1995). Biofilms associated with most surfaces are collections of microorganisms organised within an extracellular polymer matrix (Costerton 1995). Biofilms connected with medical prostheses and within host tissue tend to be monocultures, however, at other sites mixed cultures are frequently encountered. Bacteria grow as adherent biofilms within extended glycocalices, which result in chronic infections in the host especially when involved with prosthetic devices, medical implants and catheters used in patients. The make-up and cell physiology of sessile populations are substantially different from those of planktonic populations and have become problematic due to their persistence within the infected host (Fletcher 1984, Williams 1988, Gilbert *et al* 1990, van Loosdrecht 1990, Dibdin *et al* 1996). Biofilms may include mixed populations of bacterial species, along with fungi and protozoa. The metabolic capabilities of these

populations may be unrepresentative of any one member, it is also possible that mixed populations are synergistic (Gilbert *et al* 1995). In aquatic environments, sessile populations are found adherent to surfaces of sediment, stone and at the air/water interface. Biofilm formation can cause problems in many industrial processes. Biofilms form in fuel lines, pipes, cooling towers and many other sites (Costerton & Lashen 1984, Kajdasz *et al* 1984, Costerton 1995). Growth rate *per se* and nutrient limitation are important factors in bacterial pathogenesis, where the bacterial growth rate is markedly reduced from that obtained in laboratory media. Slow growing bacteria are more resistant to the effects of antibiotics and thus, infections involving bacterial biofilms are more difficult to treat due to the increased resistance of the bacteria present in the biofilm (Costerton personal communication, Costerton 1984, Costerton *et al* 1987, Kajdasz *et al* 1984, Costerton & Lashen 1984, Hewinson *et al* 1986, Brown *et al* 1988, Nichols *et al* 1989, Evans *et al* 1990a, Evans *et al* 1990b, Evans *et al* 1991, Duguid *et al* 1992, Brown & Gilbert 1993, Stewart 1994, Gilbert & Brown 1995, Suci *et al* 1994, Khardori & Yassien 1995). It is this increased resistance which causes problems and contributes to the antagonism of certain infections. The increased resistance properties must be taken into consideration when new antibiotics or biocides are being developed as the resistance in bacterial planktonic populations and the bacteria in biofilms are vastly different (Brown *et al* 1988, Hoyle *et al* 1990).

### *Planktonic Growth*

In nature, free-living or planktonic populations of microorganisms are rarely found (Gilbert *et al* 1995) as they prefer to grow in an association with surfaces in the form of biofilms (Costerton *et al* 1987). Once established as a biofilm the bacteria use the same survival strategies that they would use in a natural aquatic environment. That is, the biofilm releases 'scout' cells (planktonic) in an attempt to colonise the surrounding environment (Costerton personal communication). The responses of planktonic cells to the actions of environmental and chemical insults (including biocides, antibiotics and bacteriophages) are markedly different to those observed with sessile cells (biofilms). Kajdasz *et al* (1984) suggested that the measurement of biocidal efficiency against bacteria which exist in biofilms does not necessarily indicate the efficacy towards the same species which exist as planktonic cells. Sessile and planktonic populations require individual testing to determine their susceptibility to a specific biocide (Costerton & Lashen, 1984). Planktonic cells are

disadvantaged in comparison to the same species growing as a biofilm. The highly structured biofilm mode of growth provides bacteria with a measured homeostasis, a primitive circulatory system, a framework for the development of cooperative and specialised cell functions and a large measure of protection from antibacterial agents. Planktonic cells of the same species do not possess these advantages thus, resulting in the cells growing as biofilms, having functional predominance in natural aquatic environments (Costerton 1995).

### **Laboratory Growth**

Microbiologists inoculate fresh medium with cultures which are 16–40 hours old and often in stationary phase, which produce cell populations that are misrepresentations of populations *in vivo*. The natural ecosystems are highly competitive and require specialised adaptation for each circumstance. When *Escherichia coli* cells are starved of nutrients, they undergo physiological and genetic changes in order to maintain viability for extended time periods. These starved cells possess a growth advantage over fresh cells, which have not been exposed to nutrient deprivation. They can, when mixed with young cells, outgrow and cause the young cells death (Brown *et al* 1995). This makes the starved cells highly competitive when appropriate growth parameters resume. Cultures *in vitro* are inoculated into a chemically complex medium to achieve maximum growth. During bacterial growth there is a steady decrease in nutrient concentration. Chemically Defined Medium (CDM) is substituted in order to mimic growth parameters in nature. Growth occurs when the nutrients available support the requirements of the bacteria. When the cells are inoculated into fresh medium, they initially attenuate themselves to the nutrients available in the medium and the cells adapt their growth accordingly. For bacterial cells *in vitro*, growth is the consequence of interactions between any one of the numerous organic compounds in mineral salts medium which serves as a carbon source. The growth rate is controlled through fuelling reactions which provide precursor metabolites, reducing power and energy as ATP or equivalent (Marr 1991). All metabolism is interrelated, with a great deal of feedback, and no one reaction is the pacesetter. The physiological properties expressed by microorganisms in laboratory cultures are relevant and offer some rationale for an evolutionary process that has proceeded towards extreme species diversity and specialisation. Microorganisms display various characteristics depending upon the nature of the nutrient limiting growth and it is

imperative to recreate these conditions when attempting to mimic *in vivo* responses in the test tube. The use of CDM, is a common approach, this allows the limitation of a single nutrient, whilst the remaining composition of the medium remains the same. Due to the many responses which occur in microbial populations in nature, CDM's are useful in the task of understanding natural microbial growth (Brown & Gilbert 1995). The majority of naturally occurring bacteria exist in environments of nutrient deprivation (Brown 1977). The precise *in vivo* conditions regarding nutrient limitations of growth are poorly understood. However, the *in vivo* situation may be mimicked to produce a physiologically defined and relevant inocula once the bacterial requirements are elucidated. The studies by Klemperer *et al* (1979) suggested that the nutritional requirements for the growth of bacterial cells containing the RPI plasmid were much lower than the standard medium used for laboratory growth. The stability of the cultures containing the RPI plasmid appeared unaffected on exposure to nutritional limitation and the plasmid containing cells exhibited more efficient survival in storage.

### **Responses to Nutrient limitation**

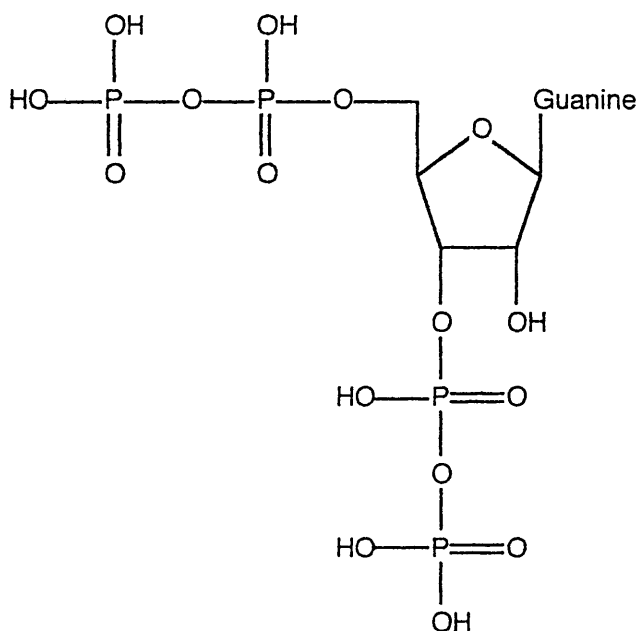
Cells have evolved many ways of surviving nutritional deficiencies, depending on whether they are sporulating (differentiating) or non-sporulating (non-differentiating) bacteria. Both types of bacteria have similar responses to nutrient limitation which result in the survival of the bacteria. Microbial differentiation is initiated due to an essential nutrient becoming growth limiting. The cell continues to synthesize new macromolecules, if there are adequate resources available for completion of the new proteins (Koch 1997). These new proteins are required for metabolism and differentiation under conditions of nutrient limitation which ensure the survival of the bacteria (Freese 1989). *Vibrio* spp. adapt physiologically and at a molecular level to nutrient and energy deprivation. These adaptations to nutrient limitation are time dependent and the responses are similar to those of *E. coli* and *S. typhimurium* (Flardh *et al* 1992, Koch 1997). Energy maintenance is essential for the protection of the cells against other bacteria, viruses and toxic chemicals etc.. *Bacillus* spp. form endospores in order to protect themselves against these adverse conditions (Koch 1997). Heterotrophic marine bacteria which are repeatedly deprived of energy or nutrients or both in their natural habitat exhibit periods of unbalanced growth. This unbalanced growth includes periods of non-growth, starvation, recovery and regrowth (Kjelleberg *et al* 1993).

In natural nutritionally suboptimal environments the bacterial populations respond to starvation by either entering dormancy (Novitsky & Morita 1977, Dow *et al* 1983, Nystrom & Kjelleberg 1989, Gilbert *et al* 1990), stringent response (Cashel & Gallant 1969, Freese & Heinze 1984, Cashel & Rudd 1987) or producing endospores (Flardh & Kjelleberg 1994, Nystrom *et al* 1990, Nystrom *et al* 1992). The morphological adaptations are minor in *E. coli* and *Vibrio* spp. but are similar to the characteristics of the true differentiating bacteria, eg: *Bacillus* spp. (Flardh *et al* 1992). The response to nutrient deprivation exhibited in *Vibrio* spp. is the shutdown of RNA, protein and peptidoglycan synthesis and the accumulation of signal molecules. The signal molecules are the highly phosphorylated nucleotides (HPNs) guanosine-3'-diphosphate-5'-diphosphate (ppGpp, Magic Spot I, MSI) and guanosine-3'-diphosphate-5'-triphosphate (pppGpp, Magic Spot II, MSII) (Fig 1). These compounds accumulate at the onset of starvation thus, indicating the onset of stringent response (SR). These responses ensure the survival of the bacteria whilst in the period of non-growth and once the required nutrients become available the starved cells resume growth and cell division (Kjelleberg *et al* 1993). The steady-state growth rate varies depending on the nutrient available for growth. The growth may be either slow or fast, depending on the growth medium but there are limits in either extreme. Growth may not always be slow in unfavourable conditions, bacteria often respond by shutting down metabolism and forming endospores, or temporarily entering a quiescent state (Koch 1997). It is critical at this point for the cell to respond rapidly to the environmental fluctuations in order to survive.

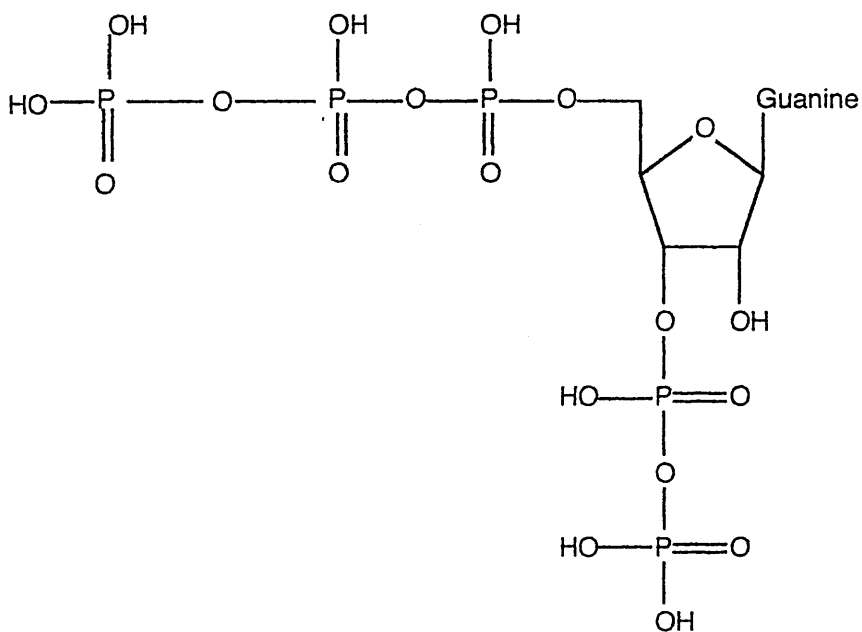
Research into the effects of amino acid limitation in bacteria has been carried out by many research groups (Block & Haseltine 1974, Lagosky & Chang 1980, McDowell *et al* 1988, Harkness *et al* 1992). The response to amino acid deprivation in non-differentiating bacteria, was found to be similar to that for sporulation in differentiating bacteria. This phenomenon was the induction of the stringent response (SR) and it is thought to ensure the bacteria's survival under nutrient limited conditions. Bacteria respond to amino acid starvation by reducing the rate of protein biosynthesis (O'Farrell 1978). There is also a lack of the corresponding aminoacylated tRNA which results in adaptations in the cell thus, inducing the SR (Ochi *et al* 1982, Rojiani *et al* 1989). Amino acid deprivation induces the *relA* gene, which codes for the enzyme ATP:GTP (GDP) 3'-pyrophosphotransferase ((p)ppGpp synthetase I) which synthesises ppGpp and pppGpp

**Figure 1:** Schematic diagram of Guanosine-3'-diphosphate-5'-diphosphate (Magic Spot I, MS I; ppGpp), and Guanosine-3'-diphosphate-5'-triphosphate (Magic Spot II, MSII; pppGpp).





**Guanosine-5'-diphosphate-3'-diphosphate  
(**'Magic Spot I'**; ppGpp)**



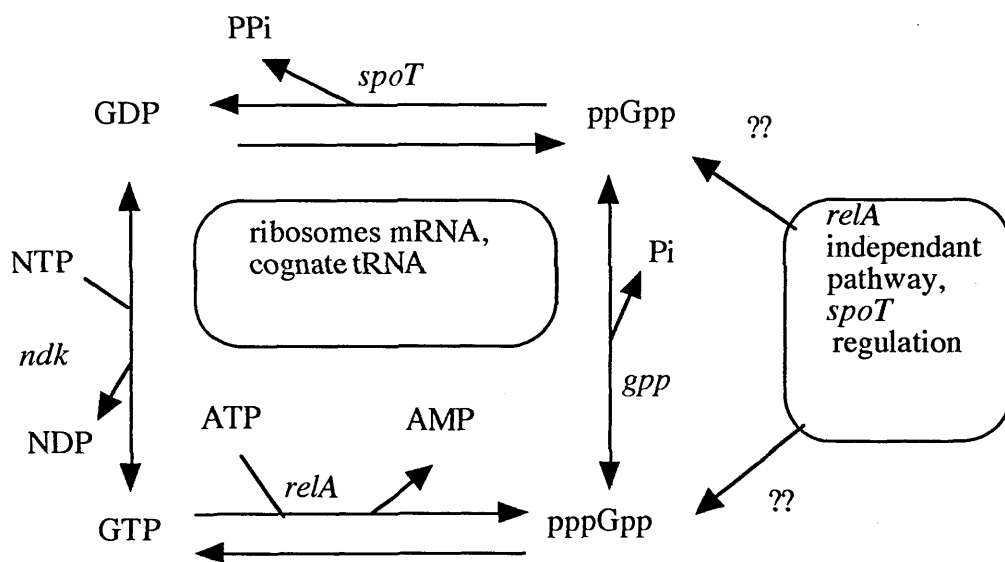
**Guanosine-5'-triphosphate-3'-diphosphate  
(**'Magic Spot II'**; pppGpp)**

during starvation (Cashel & Gallant 1969, Cashel & Rudd 1987) and is ribosome dependent (Fig 2). There is a second enzyme which is responsible for the degradation of pppGpp to ppGpp and is the product of the *spoT* gene, (p)ppGpp 3'-pyrophosphohydrolase ((p)ppGpp synthetase II). The *relA* dependent route of (p)ppGpp synthetase I synthesis has been researched extensively and is, therefore, well defined. It has been suggested that ppGpp initiates a range of metabolic changes (Gallant & Lazzarini 1976). These adaptations in the cells metabolism include the inhibition of stable RNA synthesis and the inhibition of protein synthesis (Block & Haseltine 1974, Chaloner-Larsson & Yamazaki 1975, O'Farrell 1978, Lagosky & Chang 1980, Fehr & Richter 1981(a), Kelly *et al* 1991, Gustafson *et al* 1993).

### *Dormancy*

Dormancy has been defined as a “*rest period*” or “*reversible interruption of the phenotypic development of an organism*” (Novitsky & Morita 1977). Microorganisms exhibit dormancy when deprived of their primary carbon and energy substrate. During the transition from exponential growth to stationary phase, when available nutrients are depleted, bacteria undergo a series of morphological, physical and biochemical adaptations. These changes include degradation of RNA, a reduction in ATP concentration, the expression of novel starvation proteins and an increased resistance to various stresses (Keilin 1959, Roszak & Colwell 1987, Matin 1990, Lang & Hengge-Aronis 1991, Matin 1991, Matin 1992, Siegle & Kolter 1992, Tunner *et al* 1992, Bryers & Sanin 1994). Bacteria in natural environments are subjected to periods of stress where the nutrient and energy levels are low. The consequences of these environmental conditions result in inhibition of bacterial growth which reduces the growth rate to such an extent that frequently, the growth rate approximates zero (Gilbert *et al* 1990). Cell populations which exhibit low growth rates or zero growth rates are close representations of bacteria found in oligotrophic marine environments (Morita 1988, Holmquist & Kjelleberg 1993(a), Kjelleberg *et al* 1993, Weichart & Kjelleberg 1996). It is due to the nature of the oligotrophic environment in which the bacteria exist that they have evolved complex survival mechanisms. A common response to nutrient limitation is the fragmentation of the cells, ie. they divide without growth thus, producing ultramicrocells (Bryers & Sanin 1994). Roszak and Colwell (1987) suggested that starved bacterial cells exhibit “*rapid multiple cell division*” which leads to the formation of ultramicrobacteria. The formation of these ultramicrocells is

**Figure 2:** Cellular routes of Guanosine-3'-diphosphate-5'-diphosphate (ppGpp) and Guanosine-3'-diphosphate-5'-triphosphate (pppGpp) synthesis and degradation. The enzymes involved in ppGpp metabolism are represented by their respective structural gene designations. These enzymes often have additional activities demonstrated *in vitro* which may play limited physiological roles normally, or major roles in mutant constructions. The ppGpp cycle is shown as completed by nucleoside 5'-diphosphate kinase (*ndk*), although several other enzymes have the ability to convert GDP to GTP. The enzymes of ppGpp metabolism that are now well defined are: ppGpp synthetase I (*relA*), (p)ppGpp 3'-pyrophosphohydrolase (*spoT*), pppGpp 5'-phosphohydrolase (*gpp*) and nucleoside 5'-diphosphate kinase (*ndk*) (Cashel & Rudd 1987).



the result of the completion of ongoing rounds of replication and subsequent cell division without an associated increase in biomass. The bacterial cell divides and produces two daughter cells. Which, as a result of these divisions, are reduced in cell size and exhibit a decrease in concentration of endogenous material (Schaechter *et al* 1958, Novitsky & Morita 1977, Nystrom & Kjelleberg 1989).

There are cells which, under nutritional stress, are viable but non-culturable. Jannasch (1967) suggested that below certain concentrations of substrate there were two strategies for survival

(i) the ability to grow at low substrate concentrations and

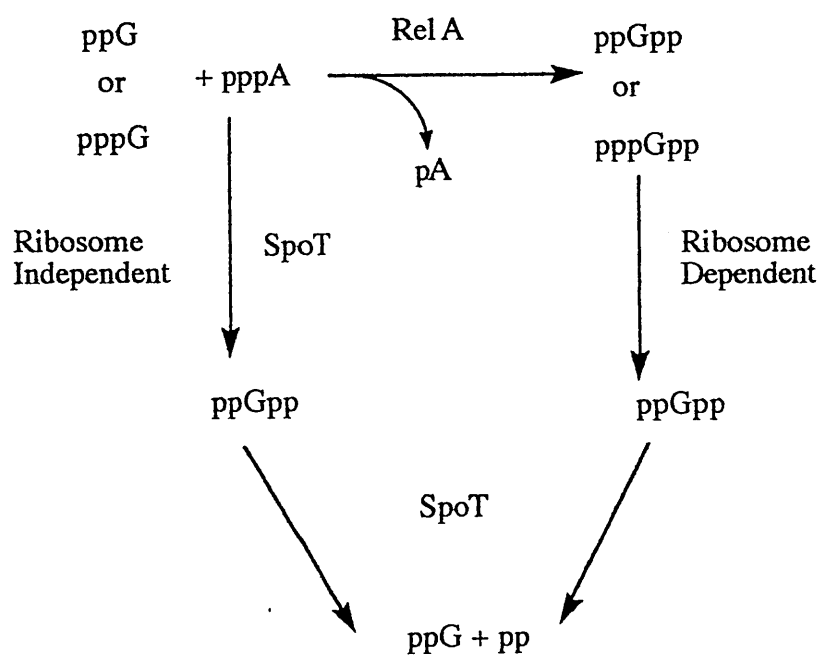
(ii) the ability to become temporarily inactive (nonculturable) and remain viable (Roszak & Colwell 1987). The enumeration of these nonculturable cells could not be carried out using conventional laboratory methods. Alternative methods were used such as uptake of radiolabelled substrate, detection of cell respiration and differential staining procedures. These methods enabled the detection and quantification of populations of viable non-culturable cells without having to grow them on standard laboratory media.

### *Stringent Response*

Non-differentiating bacterial cells possess a regulatory mechanism called “Stringent Response” (SR). The SR is a survival mechanism which is induced by nutrient limitation (Flardh *et al* 1994). The most striking property of the SR to amino acid limitation is the increase in ppGpp and pppGpp (Cashel & Gallant 1969, Pederson *et al* 1973, Richter 1976, O’Farrell 1978, Fehr & Richter 1981(a), Ochi *et al* 1982, Ruppen & Switzer 1983, Baracchini *et al* 1988, Moat & Foster 1988, Rojiani *et al* 1989) and the rapid reduction in the rate of synthesis of stable RNA (tRNA and rRNA). The production of the ppGpp and pppGpp is initiated by the *relA* gene product (p)ppGpp synthetase I which transfers phosphate molecules from the donor molecule, (ATP) to recipient molecules (GTP or GDP) (Fehr & Richter 1981(b), Beaman *et al* 1983, Cashel & Rudd 1987, Eichel *et al* 1998) (Fig 3) and is activated by the presence of mRNA and the binding of uncharged tRNA in the acceptor (A) site of the ribosome. The production of ppGpp and pppGpp has an absolute requirement for ATP and its function is the donor of pyrophosphate to either GDP or GTP (Block & Haseltine 1974). Due to addition of the pyrophosphate to the 3’ terminal of GTP during SR renders GTP unavailable for protein synthesis. Since pppGpp is a structural

analogue of GTP it is suggested that its presence on the ribosome inhibits the normal reactions of protein synthesis which utilise the guanine nucleotide (GTP) as cofactors. The formation of the initiation complex between formylmethionyl-tRNA<sub>f</sub>, 70S ribosomes, and messenger RNA depends upon the interaction between IF2 and GTP (Gallant & Lazzarini 1976). This interaction could be initiated by pppGpp as a substitute for GTP in protein synthesis, but could not be subsequently substituted in translocation. The failure of translocation of pppGpp may be due to the position of the 3'-pyrophosphate resulting in the ribosomes not being able to hydrolyse pppGpp to GTP. This phenomenon results in an overall reduction in protein synthesis. The ppGpp and pppGpp molecules are thought to be the effectors of the SR (Cashel 1969, Itikawa *et al* 1986, Cashel & Rudd 1987, Baracchini *et al* 1988). Due to amino acid limitation an uncharged tRNA attaches itself to the ribosome. This action combined with the stringent factor, produces ppGpp and pppGpp. When SR occurs there is also a concomitant reduction in the rate of synthesis of RNA, peptidoglycan, membrane material and the transport rate of particular compounds such as purines, all the result of the inhibitory action of (p)ppGpp (Freese & Heinze 1984). When observed under amino acid limited conditions *relA*<sup>-</sup> mutants do not accumulate ppGpp and pppGpp and the synthesis of stable RNA continues. Jones (1994) suggested that many cellular responses are affected by the SR and that the requirements for (p)ppGpp synthesis *in vivo* are consistent with the hypothesis that one function of the SR is to regulate the balance between stable RNA levels and the cellular demand for protein synthesis. These observations suggest that the synthesis of rRNA and tRNA are controlled by ppGpp and pppGpp (Cashel & Rudd 1987, Jones 1994). The first product of the *relA* ribosome-dependent pathway for ppGpp production *in vivo* is pppGpp. pppGpp is thought to be the precursor for ppGpp and is converted to ppGpp by (p)ppGpp synthetase II, the *spoT* gene product (Fehr & Richter 1981(a), Hara & Sy 1983). pppGpp is produced by stalled ribosomes in the presence of mRNA and uncharged tRNA (Haseltine *et al* 1972, Pederson *et al* 1973, Moat & Foster 1988). There is a synthetic route which is ribosome-independent, and involves (p)ppGpp synthetase II. The *spoT* gene product (p)ppGpp synthetase II, is also the major (p)ppGpp hydrolase and appears to be a bifunctional enzyme as it is capable of both synthesis and degradation of (p)ppGpp (Fig 3) (Moat & Foster 1988, Cashel & Gallant 1974, Jones 1994).

**Figure 3:** Stringent response. Ribosome-dependent and -independent pathways for the biosynthesis of guanosine-3'-diphosphate-5'-diphosphate. ppG (guanosine-5'-diphosphate); pppG (guanosine-5'-triphosphate); pppA (adenosine-5'-triphosphate); pA (adenosine-5'-monophosphate); ppGpp (guanosine-3'-diphosphate-5'-diphosphate); pppGpp (guanosine 3'-diphosphate-5'-triphosphate); ppG (guanosine-5'-diphosphate); PP (inorganic pyrophosphate) (Moat & Foster 1988).





When cultures of *E. coli* and *S. typhimurium* were starved of carbon their responses were similar to that for amino acid starvation, ie: the inhibition of stable RNA production and the initiation of ppGpp and pppGpp production (Fehr & Richter 1981(a), Groat & Matin 1986, Cashel & Rudd 1987). As the production of the ppGpp and pppGpp coincided with entry into stationary phase, growth of the cultures with a single substrate as the limiting factor may suggest the involvement of these compounds with the stationary phase phenomena exhibited by the cells. These phenomena include dormancy, stringent response, sporulation, development of competence, antibiotic production, chemotaxis and degradative enzyme production. These responses may occur simultaneously and require the coordinated expression of multiple gene loci (Brown 1977, Matin 1979, Jensen *et al* 1993).

Whilst nitrogen and phosphorous starvation caused the production of non-culturable and abnormal cells, these conditions of limitation failed to provoke stringency. In other organisms stringency is involved in the regulation of starvation-induced genes and optimises bacterial survival in an environment under starvation stressed conditions. In *Bacillus subtilis* the switching on of the SR can induce sporulation (Hengge-Aronis 1993). Gentry *et al* (1993) suggested that “starvation-induced synthesis of the alternative sigma factor  $s^S$ , (also designated *rpoS* or *katF*) in *E. coli* was stimulated by ppGpp and that  $s^S$  is required for survival and controls a large number of stationary-phase induced genes” (Eichel *et al* 1998). When *E. coli* cells with deletions of both *relA* and *spoT* are grown under nutrient depleted conditions, they do not synthesise any ppGpp (Xiao *et al* 1991), and fail to induce  $s^S$  production on entry into stationary phase (Gentry *et al* 1993) therefore, they lose their viability (Rojiani *et al* 1989).

### *Endosporulation*

Sporulation is not a process which a cell undertakes lightly. The cell responds to environmental signals throughout the growth cycle and, when those signals indicate the environment is becoming hostile, the cell initiates the shut down of cellular division and initiates the sporulation process. The initiation of sporulation is regulated by a series of phosphotransfer reactions, the phosphorelay signal transduction pathway (Hoch 1993a). The phosphorelay signal transduction pathway is a variant of a two-component response stimulus-response mechanism which regulates environmental responses in bacteria. The phosphate from a primary response regulator is transferred to a second response regulator by

the *spoOB* gene product phosphoprotein phosphotransferase (Hoch 1993b).

Mitchell and Vary (1989) state that sporulation of *Bacillus* spp. is generally thought to be the response to a nutrient depleted environment (Brown & Hodges 1974, Hodges & Brown 1975, Lee & Brown 1975, Al-Adham 1989). The combination of environmental and physiological signals tend to be the controlling factors in gene expression and development in both eukaryotes and prokaryotes. The Gram-positive bacterium *B. subtilis* requires certain conditions before the initiation of spore formation. These conditions include the starvation of a carbon, nitrogen or phosphorous source. Another prerequisite is that the cell density has to be fairly high and the cells must be synthesising DNA (Ireton *et al* 1993). If all these conditions are met then the cell will successfully initiate sporulation. The period between the termination of the exponential growth phase and the onset of stationary phase can be referred to as the transient state. During this period cells may still be expressing growth-related functions along side the production of new gene products which ensure survival (Dubnau 1993).

The bacterial genus *Amphibacillus*, *Bacillus*, *Clostridium*, *Desulfotomaculum*, *Oscillospira*, *Sporohalobacter*, *Sporolactobacillus*, *Sporosarcina*, *Sulfidobacillus* and *Syntrophospora* commonly form endospores (Priest 1993). The bacterial cells remain in a vegetative state as long as there are sufficient nutrients available for growth. Once any of the essential nutrients become exhausted and the cultures begin to enter stationary phase, the cells induce spore formation (Mandelstam 1969, Lopez *et al* 1979, Jeong *et al* 1990). The formation of the spore involves an observed sequence of events, both structural and biochemical in nature (Burke 1982). The resulting spore is different from the mother cell in that they have different biochemical and morphological characteristics (Foster 1956, Mandelstam 1969, Sterlini & Mandelstam 1969, Piggot & Coote 1976, Losick *et al* 1986). The levels of small molecules in spores of *Bacillus* spp. and *Clostridium* spp. were compared with levels in the spores of *Sporosarcina* spp (Loshon & Setlow 1993). The spores of *Bacillus* spp. and *Clostridium* spp. contained little or no free ATP, significant levels of ADP and AMP, high levels of 3-phosphoglyceric acid (3-PGA) and divalent inorganic cations. The spores of *Bacillus* spp. also contained high levels of free amino acids. In comparison, the spores of *Sporosarcina* spp. contained similar levels of ADP, 3-PGA, free amino acids and divalent inorganic cations, but there was no detectable levels of ATP (Loshon & Setlow 1993). This suggests that the levels of small molecules are similar in the

spores of all Gram-positive organisms and that this may reflect some fundamental and conserved feature of the process of sporulation in these organisms. The sporulation of *B. subtilis* occurs when nitrogen, carbon or phosphate are growth limiting. Abedin *et al* (1983) examined the effects of the adenosine analogues decoyinine, cordycepin and psicofuranine had upon sporulation when added to cultures containing excess levels of nutrients. They observed a decrease in the the intracellular pools of GTP and GDP and the induction of sporulation, even when there was an excess of nitrogen, carbon or phosphate, conditions which would normally prevent sporulation (Lopez *et al* 1979, Abedin *et al* 1983). The initiation of sporulation is a complicated regulatory system, requiring the cells to commit to a shut down of cell division and activate a large number of morphogenetic processes (Driks & Losick 1991, Hoch 1993a).

In response to nutrient limitation during the growth of *B. subtilis* there are a number of biochemical and morphological changes which result in the formation of an endospore. Sporulation involves the differentiation of the mother cell and the forespore. The two essential regulators of the mother-cell gene expression are *SpoIIID* gene product, a DNA-binding protein which activates or represses transcription of many different genes and  $\sigma^k$ , a subunit of RNA polymerase that directs the enzyme which transcribes gene encoding proteins that form the spore coat (Halberg & Kroos 1992). The initiation of spore formation is controlled by specific genes. The response of the cell to either continue growth or begin sporulation is regulated by the *SpoOA* transcription factor which has the capability to serve as either a repressor or an activator of transcription, depending on the target gene (Burbulys *et al* 1991). The genes which encode for competent enzymes of the phosphorelay pathway are individually regulated. The *spoOA* gene is regulated by two promoters, (i)  $s^A$  the controlling factor which is responsible for maintenance of low levels of *spoOA* protein during the exponential growth phase and (ii) the  $s^H$  promoter which initiates early sporulation at the end of the exponential phase. The phosphorylation of *spoOA* to *spoOA*-P which is essential for the initiation of sporulation is due to the actions of the *spoOB* gene product phosphoprotein phosphotransferase (Hoch 1993b).

The product of the *spoOH* gene, sigma factor  $\sigma^H$  is responsible for the transcription of *spoOA*, *spoOF*, and *kinA*, the genes required for initiation of early sporulation (Hoch 1993b). The *spoOH* gene encodes the earliest acting specific  $\sigma$  factor

which is negatively controlled by *abrB*. The *abrB* gene is involved in regulating the processes which occur at the end of the exponential phase and its main objective is to prevent the expression of post-exponential phase genes during exponential growth in nutrient sufficient conditions thus, utilising the carbon and energy available for cellular division. Under nutritionally sufficient growth conditions there are low levels of *spoOA*-P and high levels of expression of *abrB* which prevents transcription of genes encoding for proteases and some sporulating genes (eg. *spoOE* and *spoOH*), antibiotic pathways and the entire competence pathway as well as others. The inactivation of *abrB* and an increase in *spoOA*-P due to the nutrients in the medium being exhausted releases all the *abrB* regulated genes which may be expressed, if their regulatory mechanisms are activated (Strauch 1993). The concentration of  $s^H$  is controlled at the post-transcription level and increases about four-fold at initiation of sporulation. This appears to be the fail safe mechanism for the control of the  $E$ - $s^H$  regulon which is essential for the early stages of sporulation (Smith 1993).








The responses of both sporeforming and non-sporeforming bacterial species are similar when in a nutrient limited or deprived environments. This similarity may be due to both groups possessing the stringent response gene, *relA*. The activity of *relA* can induce sporulation in endosporeforming bacteria, but it is not thought to be the primary gene responsible for sporulation (Ochi *et al* 1981, Freese & Heinze 1984, Gilbert *et al* 1990). It is widely accepted that the gene responsible for sporulation is *spoOA*. The responses of these two genes appear to be very similar and their effects have been shown to be under the control of the *abrB* gene. The *abrB* gene is capable of partially suppressing the activities of both *relA* and *spoOA* genes (Gilbert *et al* 1990, Freese & Heinze 1984).

The genes involved with sporulation are transcribed at specific times during the sporulation process and, in turn, inhibit the transcription of certain vegetative genes. The transcription of RNA polymerase during sporulation is altered and mutants bearing lesions in the enzyme lose the ability to sporulate, which indicates that RNA polymerase plays a key role in this event. A rifampin-resistant mutant of *B. subtilis* sporulates poorly in Sterlini-Mandalstam (SM) sporulating medium, yet resumes the ability to sporulate on addition of the amino acids arginine, methionine, valine and isoleucine to the medium (Pun & Pennington 1980). The rifampin-resistant lesion is situated in the gene encoding RNA polymerase therefore, under nutrient limited conditions there is no enzyme production and this results in a reduced level of sporulation (Pun & Pennington 1980). These results indicated that the

enzyme RNA polymerase may regulate sporulation. Sporulation can occur in a suitable medium, 6 to 8 hours after development has been initiated at the end of the exponential growth phase (Piggot & Coote 1976, Freese & Heinze 1984) (Fig 4). The process of sporulation occurs over eight stages, each stage exhibiting specific changes to the mother cell and the development of the endospore. At the end of growth when nutrients have been depleted the cell during stage 0 is still in a vegetative state (Fig 4). The cell contains two chromosomes from the final round of vegetative DNA replication (Losick & Stragier 1992). The axial chromatin is formed due to the cohesion of the two chromosomes which forms a broad axial filament that occupies the centre of the cell during stage I and is probably linked to the membrane by mesosome attachments. The formation of a septum by membrane invagination and growth at one pole of the cell occurs during the second stage of the formation of the endospore. Freese (1972) suggested that there must be some peptidoglycan synthesis in order to give direction to the synthesis of membrane in the formation of a sheath around the axial chromatin. Thus, ensuring the subsequent formation of the septum. The formation of a free protoplast within the mother cell occurs during stage III. This is the result of the bulging of the spore septum into the cytoplasm, followed by movement of the points of attachment at the ends of the septum to the pole of the mother cell. Cortex formation and deposition of primordial germ cell wall between the membranes of the spore protoplast occurs during stage IV. The spore appears at first as a refractile object under phase contrast microscopy. Deposits of material within the double membrane of the spore protoplast forms the cortex and contains the mucopeptide of the spore. The deposition of the spore coat around the cortex occurs during Stage V. Stage VI is when the spore matures and its coat becomes denser. The spore also develops its characteristic properties at this stage. These properties include resistance to heat, antibiotics and the ability to survive for extended time periods and remain viable. During Stage VII the mother cell lyses due to lytic enzymes and the spore is then released (Mandelstam 1969, Piggot & Coote 1976, Freese & Heinze 1984).

The highly differentiated spores formed in response to nutrient stress differ in many ways from the vegetative cells. They are more resistant to environmental stresses (Zambrano & Kolter 1995). Due to the lack of endogenous metabolism the levels of compounds related to metabolism are very different to those of vegetative cells. The ATP content in dormant spores is approximately 0.4% of that in exponentially growing cells and the levels of high-energy compounds such as the majority of the sugar phosphates, reduced

**Figure 4:** Schematic representation of the morphological changes associated with the stages of sporulation (Mandelstam 1969).

						
STAGE 0 Vegetative cell	STAGE I Chromatin filament	STAGE II Spore septum	STAGE III Spore protoplast	STAGE IV Cortex formation (refractility)	STAGE V Coat formation	STAGE VI Maturation
	Antibiotic Exo-protease Protein turnover Ribonuclease Amylase	Alanine dehydrogenase	Alkaline phosphatase Glucose dehydrogenase Aconitase Heat-resistant catalase	Ribosidase Adenosine deaminase Dipicolinic acid Uptake of Ca <sup>2+</sup>	Cysteine incorporation Octanol resistance	Alanine racemase Heat resistance

pyridine nucleotides, ribonucleoside triphosphates, guanosine tetra- and pentaphosphates and tRNA are present at extremely low or undetectable levels. The absence of these high-energy compounds is compensated by the presence of the high-energy phosphate donor phosphoglyceric acid (PGA). There is enough PGA present in the dormant spore to convert the spore ribonucleotide pool to the triphosphate form by more than three fold (Table 1).

**Table 1:** Levels of several compounds in dormant spores and vegetative cells of *Bacillus megaterium* (Setlow 1975)

Compounds	Amount (nmol g <sup>-1</sup> , wet weight)	
	Dormant spores	Vegetative cells
ATP	3	725
ADP	94	-
AMP	450	-
ADP + AMP	544	195
CTP + GTP + UTP	<10	680
Total cytidine nucleotides	133 <sup>b</sup>	150 <sup>c</sup>
Total guanosine nucleotides	107 <sup>b</sup>	310 <sup>c</sup>
Total uridine nucleotides	290 <sup>b</sup>	405 <sup>c</sup>
Total deoxyribonucleotides	<1.5	181 <sup>c</sup>
PGA	6,800	-

<sup>b</sup> More than 80% as the monophosphate.

<sup>c</sup> More than 70% as the triphosphate.

- Not done.



The longevity of spores and their great resistance to adverse conditions, are coupled with their ability to germinate very rapidly in a suitable environment. These properties indicate their great importance to both the medical and food industries. Dormant spores of *B. subtilis* possess a number of properties vastly different to growing cells. These differences include increased resistance to chemicals, heat, mechanical disruption, UV irradiation and enzymes such as lysozyme (Mandelstam 1969, Freese & Heinze 1984, Nystrom *et al* 1992, Loshon & Setlow 1993, Setlow 1993, Setlow 1994, Marouga & Kjelleberg 1996). Spores are much more resistant than their growing cell counterparts to a variety of environmental stresses. The spores can remain dormant for very long periods in the absence of exogenous nutrient, even when they are in a fully hydrated environment. While there are few carefully controlled long-term studies of the latter phenomenon, survival of >80% of populations of spores of *Bacillus* spp. for periods of up to a year is not uncommon (Slepecky & Leadbetter 1983, Setlow 1992). Similarly, there are studies which indicate that spores can survive in nature for even longer periods (100-2000yrs) (Gould 1983, Slepecky & Leadbetter 1983). Recently, ancient *Bacillus* spp. spores from 25-40 million-year-old bees has been isolated from amber inclusions (Johnsonbaugh & Cano 1995). However, there are obviously no detailed population studies over such long time periods (Setlow 1994). One reason for spore survival over extended periods is undoubtedly their extreme metabolic dormancy. They neither contain nor make ATP. There is no detectable metabolism in dormant spores when they are in a nutrient limited environment. Once the environment begins to favour growth the spores begin to germinate. When germination begins there is an observed increase in ATP concentrations. This increase is due to the utilisation of the large pool of PGA stored in the dormant spore. The metabolism of PGA can only sustain ATP synthesis for a short time, then under aerobic conditions ATP is generated by oxidative metabolism of endogenous energy supplies such as amino acids or exogenous metabolites if there are any present (Setlow 1994). The formation of a bacterial endospore is a complex and sophisticated process of structural differentiation, responsible for the resistance and dormancy properties of the spore (Errington 1993). However, the success of this strategy for survival is dependent on the presence in the spore of an efficient mechanism for returning the organism to the vegetative state, initiating growth and multiplication when nutrients are available (Moir *et al* 1994). Although spores are insensitive to environmental insult, they must respond to a particular external stimuli by germinating, losing the spore structural properties that confer

dormancy and resistance (Gould 1969, Setlow 1981, Moir 1992). Germination, which may be defined as the loss of spore resistance properties, is followed by a period of outgrowth, when biosynthetic activity is resumed and an actively dividing rod-shaped cell is regenerated (Setlow 1984). The process of spore germination in the genus *Bacillus* is dependent upon the action of a germinant trigger site within the spore (Johnstone 1994). Germinants include alanine and ribosides. The spores of *B. subtilis* 168 will respond to L-alanine alone, or the combination of L-asparagine, glucose, fructose and potassium ions, none of which is germinative on its own (McCann *et al* 1996). Studies have indicated that during synchronised spore germination that RNA synthesis precedes synthesis of protein and DNA. There is an emphasis on rRNA and tRNA synthesis at the earliest stages of germination, with an increasing amount of mRNA synthesis as germination continues (Armstrong & Sueoka 1968, Margulies *et al* 1978, Setoguchi *et al* 1978).

Spores contain tRNA and ribosomes and the <sup>32</sup>P-labelling studies of Henner and Steinberg (1979) suggest that the tRNAs produced during spore outgrowth are the same as those synthesised at other stages of the life cycle. The first metabolic commitment upon germination is to produce tRNA and rRNA. Dormant spores in nature may survive for very long periods, thus, long term damage is likely. Therefore, tRNA and ribosomes may require renewal of the translational scaffolding in order to ensure future translational fidelity (Green & Vold 1993).

### **Role of MSI & MSII**

The two unusual nucleotides ppGpp and pppGpp were discovered by Cashel and Gallant (1969) in *E. coli*. The adenosine analogues, adenosine-3'-diphosphate-5'-diphosphate (ppApp) and adenosine-3'-diphosphate-5'-triphosphate (pppApp) were discovered by Rhaese *et al* (1976) in stationary phase cells of *B. subtilis*. Both ppGpp and pppGpp are associated with sporulation in differentiating bacteria and stringent response in non-differentiating bacteria (Murao *et al* 1980, Pun & Pennington 1980, Nishino & Murao 1981, Mukai 1992). Murao *et al* (1980) investigated the effects of pppApp upon the growth of *B. subtilis* IFO 3037 when exogenously supplemented to sporulating medium. The *B. subtilis* sporulated normally in the sporulation medium, but the induction of sporulation by pppApp was inhibited by the rapidly metabolizable carbon source, glucose. When grown in low-sporulating medium there was a reduction in spore production (Murao *et al* 1980). The

addition of pppApp exogenously to a low-sporulating medium deficient in  $Mn^{2+}$ , stimulated spore production by about 100-fold. The addition of pppApp to low-sporulating medium did not affect the growth rate of the cells. The supplementation of pppApp at 0 time to this medium did not induce premature sporulation of the cells, as heat resistant spores appeared after 24 hours of growth. If pppApp was capable of the early induction of sporulation, it would have occurred before this time. The incorporation of pppApp into the cell was investigated using  $^{14}C$ -pppApp and only 2.7% of total of applied radioactivity was found inside the cells (Murao *et al* 1980). Thirty percent of the  $^{14}C$ -pppApp had been degraded to smaller molecules which corresponded to AMP or ADP when the culture filtrate was analysed using PEI-cellulose thin layer chromatography. These results suggest that pppApp is not incorporated into the cell, and that the smaller degradation products, AMP and ADP, may be more easily incorporated into the cell than pppApp. Rhaese *et al* (1976) suggested that the role of pppApp was different from that of ppGpp and pppGpp, as the activity of the pppApp appears to be outside the cell, due to the extremely low levels found inside the cell. It was suggested that the pppApp may be involved in the control of amino acids due to the increase in sporulation when pppApp was added to medium containing casamino acids. Nucleotides such as ATP have a chelating function and since pppApp have 2 or 3 phosphates in the 3' and 5' positions, this may increase the chelating ability of the nucleotide. It may be possible that the presence of  $Mg^{2+}$  or  $Ca^{2+}$  could counteract the effects of exogenously added pppApp. This area still requires further experimental work to fully explain the mode of action of pppApp (Murao *et al* 1980).

In *B. subtilis* sporulation occurs due to biochemical and genetic alterations and is controlled by the transcription of specific genes at particular times throughout the process. There are many metabolic interactions which are responsible for the formation and degradation of cellular components. Amongst these reactions is a simple mechanism which regulates protein synthesis in response to the demands of the cell. The regulation of these cellular requirements is mediated by ppGpp and pppGpp (Cashel 1975, van Ooyen *et al* 1976). Early studies disclosed that ppGpp inhibits a step in the initiation of protein synthesis *in vitro* (Rojas & Ehrenberg 1991). The codon responsible for the binding of the charged tRNA is inhibited by both GDP and ppGpp. Due to the much higher levels of ppGpp than GDP, during stringent response, it is thought that *in vivo*, accumulation of ppGpp affects

initiation and elongation, which both requires GTP (Moat & Foster 1988). During SR in bacterial cells the regulatory nucleotide ppGpp, and its precursor pppGpp regulate the transcription of various genetic units as well as controlling important enzymes involved in metabolic pathways. ppGpp is produced by a series of phosphoryl and pyrophosphoryl additions and hydrolysis reactions. *In vivo* studies have disclosed that the regulation of the ppGpp cycle is at the two reactions involving pyrophosphates (reactions 2 and 4) (Equation 2; Hara & Sy 1983).

(Equation 2)



Both pppGpp and ppGpp are synthesised rapidly during the stringent response. The enzyme responsible for this phenomenon is pppGpp synthetase I, the product of the *relA* gene, and its activity is influenced by the presence of a ribosome-mRNA complex containing a codon specific uncharged tRNA (Hara & Sy 1983). In *relA*<sup>+</sup> *E. coli* cells, pppGpp and ppGpp rapidly accumulate on inhibition of protein synthesis, as a result of amino acid starvation or limitation of aminoacylation of tRNA. On addition of amino acid to the bacterial growth medium, the ppGpp and pppGpp concentrations rapidly become depleted, thus, initiating the synthesis of RNA and protein production. *relA*<sup>-</sup> mutants which have exhausted the amino acid supply fail to accumulate RNA or HPN's. These findings suggest the role of HPN's appears to be the regulation of RNA synthesis (Hara & Sy 1983). There is a second gene which is involved with the metabolism of ppGpp and pppGpp, the *spoT* gene. In *spoT*<sup>-</sup> stringent strains, there is no accumulation of pppGpp, but they accumulate far more ppGpp than *spoT*<sup>+</sup> stringent strains on deprivation of amino acids. It is thought that *spoT* gene is involved in the transformation of ppGpp to pppGpp as in *spoT*<sup>-</sup> *relA*<sup>+</sup> strains the rate of ppGpp deterioration was much slower than that in strains containing *spoT*<sup>+</sup> *relA*<sup>+</sup>. It was also observed that the addition of the deprived amino acid to the culture of the *spoT*<sup>-</sup> *relA*<sup>+</sup> strain resulted in the accumulation of RNA at a slower rate than in the *spoT*<sup>+</sup> *relA*<sup>+</sup> strain

(Chaloner-Larsson & Yamazaki 1975).

One of the functions of ppGpp is to protect the bacteria by inhibiting protein synthesis. This function prevents mistranslation of proteins synthesised due to errors at the amino acid starved codons. It is therefore, assumed that the production and role of ppGpp prevents these high levels of errors by inhibiting overall protein synthesis (Rojas & Ehrenberg 1991). Hamel and Cashel (1973) investigated the role of pppGpp in protein synthesis by ribosomes of *E. coli*. They suggested that if the reaction was catalysed by initiation factor 2 (IF-2) and elongation factor T (EF-T) that pppGpp could be substituted for GTP completely. They found that pppGpp was as effective as GTP in catalysing the IF-2-dependent binding of fMet-tRNA to the ribosomes and thus, the formation of *N*-formylmethionylpuromycin and EF-T-dependent ribosomal binding Phe-tRNA resulting in the formation of *N*-acetyl-phe-Phe-tRNA. Therefore, pppGpp could be substituted for GTP in protein synthesis, but it could not substitute GTP in the translocation reaction. It was thought that this was due to the ribosomes being unable to hydrolyse pppGpp to GTP. They suggested that the position of the 3'-pyrophosphate caused the inhibition of translocation.

### **Antibiotic production**

A number of Gram-positive bacteria that inhabit complex ecological communities such as those in soil and aquatic environments, produce an abundance of special compounds that are believed to enhance their survival capabilities (Bu'lock 1961, Schaeffer 1969, Katz & Demain 1977, Nakano & Zuber 1990). These special compounds (secondary metabolites) are produced under conditions of nutritional stress and are accumulated in stationary-phase cultures (Schaeffer 1969, Katz & Demain 1977). It is believed the function of these secondary metabolites is to help the organism compete for limited resources, thus enhancing the producer organisms survival (Katz & Demain 1977, Bennett & Bentley 1989, Vinning 1990). The secondary metabolites produced by Gram-positive bacteria include many antibiotics. The antibiotics synthesised include  $\beta$ -lactams, aminoglycosides, polypeptides and small polypeptides.

One of the most important bacterial genera involved in the production of secondary metabolism belongs to the Actinomycete family, the *Streptomyces* spp. This genus produce numerous antibiotics by secondary metabolism and is therefore, of particular interest to the researchers working in this area. The genes involved in antibiotic production

have been identified and characterised. They were found to be clustered together in a series of contiguous operons which occupied from around 15kb to as many as 100kb. Included within the clusters were pathway-specific regulatory genes and genes encoding resistance to the antibiotic produced by the bacteria. Many of the *Streptomyces* spp. produce autoregulators, such as the A factor (2*S*-isocaprolyl-3*S*-hydroxymethyl- $\gamma$ -butyrolactone), produced by *Streptomyces griseus*, which is required for the production of streptomycin and aerial spores. Mutants which have no A factor or have had the production of A factor inhibited cannot produce the antibiotic or the aerial spores, but exogenous addition of A factor restores both phenotypes. The involvement of ppGpp and pppGpp on the differentiation and secondary metabolism, may affect both antibiotic and A factor production (Ochi 1987). It was concluded that ppGpp, produced in response to nutrient limitation, may be an early signal in *S. griseus* and may induce the synthesis of streptomycin.

Ochi (1987) isolated a relaxed mutant of *Streptomyces antibioticus* designated *relC49* which accumulates much lower levels of ppGpp than the parent strain *Streptomyces antibioticus* IMRU 3720. The maximum level of ppGpp in *relC49* was approximately 25% lower than the level observed in *S. antibioticus* IMRU 3720. *S. antibioticus* IMRU 3720 exhibited a burst of ppGpp synthesis between 18 and 24 hours of growth and this coincided with the synthesis of actinomycin. These findings indicated that ppGpp may be a regulator of antibiotic synthesis (Kelly *et al* 1991). There are three enzymes involved in actinomycin synthesis in *S. antibioticus* these are phenoxazinone synthase (PHS) which also inhibits the increase of the activity of the enzyme 3-hydroxyanthranilate-4-methyltransferase (HAMT) and actinomycin synthase (ASI). The enzyme HAMT displayed relatively constant levels of activity in both *relC49* and IMRU 3720, whereas the second enzyme ASI, which is abundant in IMRU 3720, was undetectable in *relC49*. The PHS levels were significantly reduced in *relC49* when compared to IMRU 3720, the levels of PHS in *relC49* never exceeded 17% of the maximum levels observed in IMRU 3720. Kelly *et al* (1991) suggested from these results that ppGpp may control the transcription of the genes responsible for PHS and ASI production, as the levels of these enzymes were either reduced or undetectable. HAMT levels however, remained relatively constant over the experimental series thus, indicating that the ppGpp controls the transcription genes for PHS and ASI synthesis, but not for HAMT.

Ochi and Ohsawa (1984) used *Bacillus subtilis* Marburg strain 61884 for their

studies as it produces many peptide antibiotics. They investigated the involvement of SR in antibiotic production due to amino acid limitation. The synthetic medium used for bacterial growth contained excess glucose, ammonium ions, phosphate, 20mM L-glutamate and 20mM L-aspartate. The antibiotic production occurred during exponential growth phase in this medium and ceased at the onset of stationary phase. They suggested that this was due to the growing culture having to synthesise the amino acids present *de novo*, resulting in a reduced pool of intracellular amino acids when compared with bacteria growing in a complete medium (Ochi & Ohsawa 1984). The pattern of antibiotic production was altered when the synthetic medium was supplemented with casamino acids. The synthesis of antibiotics occurred only at the end of exponential growth phase, once the casamino acids had been utilised. The level of antibiotic production was approximately one third of the amount when no casamino acids were present. The effects of SR upon antibiotic production and sporulation were studied using *relA*<sup>-</sup> and *relA*<sup>+</sup> strains. They were grown in two different types of growth conditions. One medium contained sufficient levels of amino acids to support growth and the second medium contained amino acids in limiting concentrations. The amino acid limited medium resulted in an increase of antibiotic production in the *relA*<sup>+</sup> strain but decreased production in the *relA*<sup>-</sup> strain, when compared to growth in synthetic medium. From these results it would indicate that antibiotic production is controlled by SR and not the effects of amino acid limitation (Ochi *et al* 1981). They proposed that the production of (p)ppGpp during amino acid deprivation induces secondary metabolism by a direct molecular mechanism (Ochi & Ohsawa 1984).

*B. subtilis* produces several antibiotics, these are predominantly cyclic peptides and antifungal antibiotics. The peptide antibiotics are composed of amino acids but they rarely show any similarity to the gene-encoded polypeptides with respect to their structure and mechanisms of biosynthesis (Zuber *et al* 1993). The peptide antibiotics often undergo post-translational processing and modification before they are transported out of the producing cell. The modifications include N-methylation, acylation, glycosylation, racimization from L to D forms and covalent linkage to a variety of functional groups including nucleosides (Kleinhauf & von Dohren 1988, Martin & Liras 1989, Kleinhauf & von Dohren 1990, Nakano & Zuber 1990). The modifications occur in order to produce a mature product (Schnell *et al* 1988), and can activate the production and incorporation of

over 300 different constituent compounds (Kleinhauf & von Dohren 1990). Some of the antibiotics produced by *B. subtilis* include alboleccitin (antifungal); bacillomycin (antifungal); bacilycin (antifungal and antibacterial); subtilin (antibacterial) and surfactin (antimycobacterial and membrane acting).

### **Antibiotic resistance**

Antibiotic resistance is a major public health problem, research and development of new drugs to overcome these problems costs the USA \$100 million per year (Niemeyer 1994). There are two general types of antibiotic resistance intrinsic and acquired.

#### *Intrinsic resistance*

Intrinsic resistance is the result of the response of the cells to exposure to the action of the antibiotic and is usually expressed by chromosomal genes. Intrinsic resistance is common to the majority of isolated resistant strains. It is associated with the 'normal' genetic constituents of the cell. The methods of intrinsic resistance include impaired uptake and the absence of an enzyme or metabolic interaction (van Asselt *et al* 1995). The main mechanism of intrinsic resistance is due to the impermeability of the cells outer membrane (Nichols 1989, Nichols & Hewinson 1989, Russell & Chopra 1990, Duguid *et al* 1992, Niemeyer 1994, Fukuda *et al* 1995, Dibdin *et al* 1996, Kohler *et al* 1996). The failure of the antibiotics to penetrate the outer membrane is due to their high molecular weight and/or hydrophobic properties. If the molecular weight is above approximately 600 daltons they are prevented from entering the porins formed by the outer membrane proteins (OMP's). The replacement of phospholipid in the outer membrane with lipopolysaccharide (LPS) which is resistant to hydrophobic antibiotics prevents the antibiotics crossing the outer membrane to reach their target. This probably occurs due to the rigid structure of LPS and the strong interaction between the antibiotic and the LPS molecules (Hewinson *et al* 1986, Russell & Chopra 1990).

#### *Acquired resistance*

Acquired resistance is developed when strains emerge from a previously sensitive bacterial population, normally after exposure to the antibiotic agent. The acquisition of plasmids and transposons or chromosomal mutations is the usual methods of acquired



resistance (Russell & Chopra 1990). Chromosomal mutation is a well established method of antibiotic resistance. These mutations involve changes in the base sequence of DNA which fall into two broad categories;

(i) Microlesions: this comprises an adaptation in one base pair which may be the transition, transversion or frameshift mutation. Transition mutations consist of the replacement of one purine for another purine, thus resulting in the substitution of one pyrimidine for another pyrimidine. Transversion is the substitution of a purine for a pyrimidine and *vice versa*. The deletion or addition of one or two base pairs results in a frameshift mutation.

(ii) Macrolesions: this involves far more extensive adaptations and can comprise of deletions, duplications, inversions or translocations (insertions). This type of alteration is far more severe. Theoretically, any type of chromosomal mutation may directly or indirectly confer antibiotic resistance on the bacterial cell. Plasmids are extrachromosomal genetic elements which replicate independently of the chromosome and, frequently, carry the genes which confer antibiotic resistance.

Plasmid determined resistance is more common than chromosomal resistance. This is due to specific factors which are (i) in the absence of antibiotic selection pressure, only a few cells of the bacterial population are required to maintain plasmids. This reduces the biochemical stress on the cell which would be involved during the replication and expression of plasmid DNA. The maintenance of plasmids in certain cells ensures that selective pressure after exposure to antibiotics results in the survival of the plasmid carrying (resistant) progeny which is then able to replicate. (ii) The genes encoded by the plasmid are essentially more motile than chromosomal genes due to the ability of the plasmid to transfer within and between certain species. Plasmids are transferred by either conjugation or transduction. Conjugation requires cell-to-cell contact thus, the DNA is transferred by the donor bacterium to the recipient. This ability to conjugate is encoded by conjugative plasmids of which many also confer antibiotic resistance. Transduction is the transfer of genetic material from one bacterium to another through the use of a bacteriophage. The gene or genes of one (host) bacterial cell become incorporated in phage particles which, after release from the dead host, act as vectors in transport of this genetic material to other bacterial cells. There are many biochemical mechanisms for antibiotic resistance which have been summarised in Table 2 (Russell & Chopra 1990).

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**Table 2:** Mechanisms of antibiotic resistance (Russell & Chopra 1990)

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Mechanism	Example
1. Alteration (antibiotic inactivation)	Acquired resistance to aminoglycosides, beta-lactams and chloramphenicol. Intrinsic resistance to beta-lactams.
2. Target site in the cell is insensitive to the inhibitor but still able to perform its normal physiological function.	Acquired resistance to aminoglycosides, beta-lactams, quinolones, rifampicin and trimethoprim. Intrinsic resistance to trimethoprim.
3. Decreased antibiotic accumulation	
(a) Impaired uptake	Intrinsic and acquired resistance to many antibiotics.
(b) Enhanced efflux	Acquired resistance to tetracycline.
4. By-pass of antibiotic-sensitive step by duplication of the target site, the second version being insusceptible to drug action.	Acquired resistance to methicillin, sulphonamides and trimethoprim.
5. Overproduction of target so higher antibiotic concentrations are needed to inhibit bacterial growth.	Acquired resistance to trimethoprim.
6. Absence of an enzyme/metabolic pathway.	Intrinsic resistance to some antibiotics in certain species.

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Aminoglycosides are commonly used for many serious infections, but due to their over use, bacteria have built up resistance to their action. Gram-negative bacteria have become resistant

to many aminoglycosides due to the modification of enzymes. These modifications inhibit the binding of the antibiotics to their ribosomal target and prevents their uptake by the cell. The three enzymes and their encoding genes involved in the inhibition of the binding of the aminoglycosides are aminoglycoside acetyltransferase AAC(6') (*aac*), aminoglycoside phosphotransferase APH(2'') (*aph*) and adenylyltransferases (ANT's) (*ant*). The resistance of aminoglycoside antibiotics is achieved by modification of the antibiotic during transport into the cell. This results in the resistance to a particular antibiotic being determined by competition between the rate of drug uptake and drug modification. If the rate of uptake of the drug is greater than the rate of modification, the active drug reaches the ribosomes and inhibits protein synthesis. Whereas, if the rate of drug modification is greater than the rate of transport, it is the modified drug which is accumulated. There is no subsequent inhibition of protein synthesis and the cells become resistant. Aminoglycoside resistance was found to be transferable from clinical isolates to *E. coli* 3110 rifampicin resistant strain, the recipient transconjugate bacteria (Russell & Chopra 1990, Kallova *et al* 1995, Vanhoof *et al* 1995).

Macrolide antibiotics are inhibitors of protein synthesis and are generally bacteriostatic, but at high concentrations they can become bactericidal. There are three different chromosomal mutations in *E. coli* that have the ability to confer resistance to macrolide antibiotics. The mutant designated *eryA* exhibited an alteration in the protein L4, a large protein synthesised by the 50S ribosomal subunit (Russell & Chopra 1990). The alteration results in the loss of macrolide binding to the ribosomes. It was deduced from this that *eryA* locus may also be the structural gene responsible for synthesis of the L4 protein. The second type of mutant, designated *eryB*, confers a change in the L22 protein, but the *eryB* locus does not appear to be the structural gene for L22. *eryC* is the third mutant, and appears to be responsible for the maturation of the ribosomal RNA and a 30S subunit protein. The mechanisms of resistance conferred by the two mutations *eryB* and *eryC* are unclear. Macrolide antibiotics exhibit selective toxicity to the 50S subunits of the ribosome. Once the antibiotic has bound to the 50S ribosomal subunits it stimulates the dissociation of peptidyl-tRNA from the ribosomes during translocation thus, inhibiting protein synthesis. The direct binding to the 23S ribosomal RNA has been observed, but due to alterations in the large protein L4, the ability to bind the antibiotic at this site is lost. Resistant strains of *E. coli* possess mutations which affect the ribosomal protein L4 or L22. It is this mutation which is thought to be the cause of the resistance.

Beta-lactam antibiotics possess three mechanisms which confer resistance: (i) the outer membrane of Gram-negative bacteria becomes impermeable to the antibiotic; (ii) the production of the enzyme  $\beta$ -lactamase and (iii) modification of one or more penicillin binding proteins. These mechanisms can occur separately or together and result in the acquired resistance to beta-lactams. The stringent response is also involved in the regulation of bacterial envelope synthesis, this includes the penicillin binding proteins (PBPs) which are proteins responsible for the structure of the peptidoglycan layer of the bacterial cells. Bouloc *et al* (1992) suggested that *E. coli* is permanently resistant to mecillinam under conditions of stringent response and it is due to the high ppGpp pools which block PBPs.

Ishiguro and Ramey (1976) found that ppGpp was a negative effector of some of the steps in peptidoglycan synthesis and is involved in autolysin synthesis (Goodell & Tomasz 1980, Vanderwel & Ishiguro 1984). Bouloc *et al* (1992) proposed that ppGpp alters the cells requirement for penicillin-binding protein 2 (PBP2). PBP2 is essential for the maintenance of the rod shape of *E. coli*. Inactivation of the PBP2 results in the formation of spherical cells which normally stop dividing, they increase in size and volume then ultimately die. This lethal action is avoided in certain cells due to mecillinam resistant mutants possessing a partially defective aminoacyl-tRNA synthetase, which induces a high pool of ppGpp. This partial induction of stringent response is responsible for the cells ability to tolerate PBP2 inactivation. PBP2 is an essential protein whose inactivation results in blockage of some vital process apart from cell division, but a high pool of ppGpp prevents this blockage, by either interrupting the communication between PBP2 and the vital process concerned, or bypassing the vital process itself (Bouloc *et al* 1992, Gustafson *et al* 1993). The resistance of *E. coli* to mecillinam was observed in the presence of certain amino acids and the partial deprivation of isoleucine and/or valine induced the stringent response. Further work determined which amino acids from leucine, serine, serine + methionine + glycine (SMG) and cysteine conferred resistance to mecillinam and ampicillin. Cysteine was the only amino acid which protected the cells from both antibiotics.

Both Gram-positive and Gram-negative bacteria have the ability to develop resistance to the antibiotics which belong to the quinolone group. The resistance is associated with mutations in chromosomal genes (Russell & Chopra 1990). Quinolone antibiotics antagonise the activities of DNA gyrase, including the introduction of negative supercoiling, catenation and decatenation, and unknotting (Gellert *et al* 1983, Wang 1985,

Wang 1987, Drlica 1992, Drlica & Zhao 1997). In Gram-negative bacteria the gene *norA* or *gyrA* mutations produce alterations in the A subunit of DNA gyrase which results in resistance to the quinolone nalidixic acid. The *gyrA* mutations are point mutations arising from transitions or transversions and were found to be clustered at the amino-terminal end of the *gyrA* protein subunit (Yoshida *et al* 1988, Yoshida *et al* 1990, Drlica & Zhao 1997). This protein subunit is close to the DNA binding-site of the subunit. It is hypothesised that the mutations result in a conformational adaptation in the DNA gyrase after the association of the A and B subunits to form the functional enzyme. The conformational adaptations could result in a decrease in binding of the nalidixic acid to the gyrase enzyme (Shen *et al* 1989a, Shen *et al* 1989b, Russell & Chopra 1990). This mutation does not confer resistance to the newer quinolones such as ciprofloxacin, norfloxacin and ofloxacin. The gene responsible for conferring resistance to nalidixic acid and the newer quinolones is *gyrB*. There are two *gyrB* mutants which are the result of a transition mutation (Yamagishi *et al* 1986). The *gyrB* products possess an electrical charge which is affected by both mutations, thus indicating that the antibiotic gyrase interaction is influenced by this charge. They both affect the binding of the quinolones to the DNA gyrase B subunit as a result of the electrostatic repulsion of the *gyrB* product.

## Summary

In summary, the literature review suggests that the environmental stresses imposed upon natural populations of bacteria in various ecosystems result in drastic adaptations in order to ensure the survival of these organisms. The main stress encountered by the bacteria is nutrient limitation. The response to nutrient limitation is the induction of either dormancy, stringent response or sporulation. During these responses many biochemical adaptations occur, such as the production of guanosine-3'-diphosphate-5'-diphosphate and guanosine-3'-diphosphate-5'-triphosphate, the signal molecules for stringent response induction (Murao *et al* 1980, Pun & Pennington 1980, Nishino & Murao 1981, Mukai 1992). These signal molecules appear as the growing culture enters stationary-phase. Once the bacterial cells have entered stationary-phase the cells produce many secondary metabolites. Particular to Gram-positive bacteria is the production of antibiotics, which are believed to enhance the survival of the bacteria involved (Katz & Demain 1977, Bennett & Bentley 1989, Vinning 1990). The formation of bacterial endospores confers

resistance to many environmental factors such as heat, antibiotic resistance and enhances the long-term survival of the spore. The spore retains the ability to germinate rapidly once the environment provides the nutrients required for growth. The daughter cells produced from these spores carry the resistance properties conferred upon the parent cell they were produced from (Russell & Chopra 1990). The ability to retain these resistance properties can be problematic, particularly if the resistance is to antibiotics. The cellular adaptations which occur due to the nutritional limitation and the production of secondary metabolites at the onset of stationary phase suggest that there may be a link between the production of HPN's and the bacterial resistance to antibiotics. Both bacterial resistance and production of these novel compounds occur during nutrient limitation and therefore, may be interrelated.

### **Objectives of this study**

The objective of this study is to determine if ppGpp and pppGpp production can be identified and used as an indicator of bacterial resistance to antibiotics and antimicrobial agents. The project also aims at an understanding of how bacteria respond to specific nutrient limitations and exposure to antibiotics under these conditions.

## GENERAL EXPERIMENTAL METHODS

### Culture Media

#### *Organisms and Culture Maintenance*

The organisms *Streptomyces adaphospholyticus* A-4668 NCIMB 13429, *Escherichia coli* NCIMB 10000 and *Bacillus subtilis* NCIMB 12900 were obtained from the National Collections of Industrial and Marine Bacteria (NCIMB), Aberdeen, UK. *S. adaphospholyticus* was revived and maintained on tryptone soya agar slopes (TSA) (Oxoid CM 131) in quadruplicate. Inoculated tryptone soya agar slopes were incubated at 28°C for 48 hours (Nishino & Murao 1974). *E. coli* and *B. subtilis* were maintained on nutrient agar slopes (Oxoid CM3) in quadruplicate. Inoculated agar slopes were incubated overnight at 37°C. The four slopes were numbered from 1 to 4. Slope number 1 was used to inoculate overnight cultures for experimental purposes and slope number 2 was the backup slope in case of contamination. Slope number 3 was used for further subculturing and slope number 4 was kept as part of a refrigerated stock culture collection. The maintenance of cultures in quadruplicate ensured that the stock bacterial cultures were not ruined by a single occurrence of contamination. After the incubation period, stock cultures were maintained at room temperature in a darkened cupboard. Culture maintenance was achieved by subculturing onto fresh nutrient agar slopes at fortnightly intervals.

### Gram Stain

Gram stains were performed before subculturing bacteria to ensure purity of the cultures. A loopful of culture was aseptically removed from stock culture 3 and placed on to a microscope slide with a drop of water. This was spread evenly over the surface of the slide and left to air dry for 30 minutes. The slide was then heat fixed by passing it rapidly, three times through a Bunsen flame. The slide was flooded with the crystal violet and left for 1 minute. The stain was decanted off and the slide was then flooded with Lugol's iodine, a mordant, and left for 1 minute. The slide was then held at an angle of 45° next to running water and rinsed three times with decolouriser acetone/alcohol, and then placed immediately under the running water. The slide was then counterstained with safranin and left for 4-5 minutes. The slide was then rinsed off with running water and blotted twice to dry (Tortora *et al* 1992). The prepared slide was then viewed under an oil immersion microscope (x1000)

to determine the culture purity.

### **Reagents and Equipment**

Aldrich, Gillingham, Dorset: Sodium citrate; Magnesium chloride ( $\text{MgCl} \cdot 6\text{H}_2\text{O}$ ); Ferric chloride ( $\text{FeCl} \cdot 6\text{H}_2\text{O}$ ); Manganese chloride ( $\text{MnCl} \cdot 4\text{H}_2\text{O}$ ); Calcium chloride ( $\text{CaCl}_2$ ); Glycerol; Iron(II) sulphate heptahydrate ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ); Manganese sulphate hexahydrate; Glycine; Chloroacetaldehyde ( $\text{ClCH}_2\text{CHO}$ ); Terbium(III) nitrate pentahydrate ( $\text{Tb}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ ); Maleic acid; Sodium nitrate ( $\text{NaNO}_3$ ).

BDH Chemicals, Poole, Dorset: Disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ); Potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ); Dipotassium orthophosphate ( $\text{K}_2\text{HPO}_4$ ); Ammonium chloride ( $\text{NH}_4\text{Cl}$ ); Glycerol; Ethanol; Sodium chloride; D-Glucose; Calcium chloride ( $\text{CaCl}_2$ ); Magnesium sulphate heptahydrate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ); Phosphoric acid ( $\text{H}_3\text{PO}_4$ ); Sodium formate ( $\text{HCOONa}$ ); Perchloric acid; Sodium sulphate ( $\text{NaSO}_4$ ); Sodium bisulphite ( $\text{NaHSO}_4$ ); Chloroform; Nitric acid; Decon 90.

Fisons, Loughborough, England: Potassium hydroxide ( $\text{KOH}$ ); Methanol (HPLC grade); Hydrochloric acid ( $\text{HCl}$ ); Sulphuric acid ( $\text{H}_2\text{SO}_4$ ); 1-Amino-2-naphthol-4-sulphonic acid ( $\text{NH}_2\text{CH}_10\text{H}_5(\text{OH})\text{SO}_3\text{H}$ ); Sodium sulfite ( $\text{NaSO}_3$ ); Potassium dihydrogen phosphate HPLC grade ( $\text{KH}_2\text{PO}_4$ ); Sodium sulphate analar ( $\text{NaSO}_4$ ).

Hewlett and Packard. USA: Hewlett & Packard HP3396 Series 911 Integrator; Hewlett & Packard Series 1050 UV Detector.

Hichrom Ltd. Theale, Reading, Berks: HPLC Partisil SAX [Whatman],  $\text{SO}_4^{2-}$  form column.

Hook and Tucker Instruments Ltd. Croydon, England. Rotamixer 7930

Hopkins & Williams Ltd., Chadwell Heath, Essex: Formic acid: Ammonium molybdate.

Kyowa, Tokyo, Japan. Kyowa microscope 765917.

Oxoid, Basingstoke, Hants: Polypeptone (L37), Tryptone soya agar (CM 131), Nutrient Agar (CM3) .

Pharmacia Biotech St. Albans, Herts: FPLC equipment, Pump P-500; Gradient programmer GP-25; Single path monitor UV-1 optical unit; Mixer 5MPa; Fraction collector FRAC-100 &



Mono S column.

Sigma, Poole, Dorset: Trizma hydrochloride; Luciferin-luciferase firefly lantern extract; Adenosine triphosphate (ATP); Adenosine diphosphate (ADP); Adenosine monophosphate (AMP); Guanosine triphosphate (GTP); Guanosine diphosphate (GDP); Guanosine monophosphate (GMP); Lithium chloride (LiCl); L-Tryptophan; Potassium chloride (KOH); Sodium hydroxide (NaOH); PEI-cellulose thin layer chromatography plates (20cmx20cm); Erythromycin; Streptomycin; Ampicillin; Nalidixic acid; Coomassie brilliant blue R-250; Bovine serum albumin.

Spectraphysics San Jose, CA. USA : Spectraphysics SP8800 Ternary HPLC Pump, Supelco, Poole, Dorset: HPLC Supelcosil LC- 18-T column.

#### *Deionisation of Glassware.*

All glassware was washed then soaked overnight in 5% Decon 90 (BDH), rinsed in deionised water, and then soaked for a least one hour in 1% HCl. The glassware was then rinsed six times in deionised water (Lee *et al* 1982) then dry sterilised at 160°C for 90 minutes.

#### **Sterilisation and preparation of media**

Nutrient agar (Oxoid CM3) was obtained from Oxoid, Basingstoke, Hants. Preparation was by the manufacturers instructions and sterilisation was carried out by autoclaving at 121°C, 15psi for 15 minutes.

#### *M9 Medium*

Aliquots (100mL) of M9 salts (10-fold concentration) (Koch & Gross 1979) were dispensed into Erlenmyer flasks (200mL) and autoclaved at 121°C, 15psi for 15 minutes. Aliquots (50mL) of each of the following solutions were prepared in deionised water and dispensed into Erlenmyer flasks (100mL); 20% w/v glucose, 0.1M magnesium sulphate and 0.01M calcium chloride. These were autoclaved at 121°C, 15psi for 15 minutes. M9 medium was prepared according to the protocol of Koch & Gross (1979).

*Leitch & Collier CDM (L&C CDM)*

L&C CDM was prepared according to the instructions in Table 3 (Leitch & Collier 1996). Chemicals were dissolved in order and the pH was adjusted to 6.5 with the addition of 1M sodium hydroxide. The L&C CDM was filter sterilised through a sterile 0.22µm nitrocellulose filter (Whatman). The medium (25mL aliquots) was dispensed aseptically into sterile deionised Erlenmyer flasks (100mL). These protocols were repeated when nutrient limited media were prepared (Tables 4 & 5).

**Table 3:** Composition of Leitch & Collier  
Chemically Defined Medium  
(Leitch & Collier 1996).

Component	gL <sup>-1</sup>
Glucose	4.000
Sodium citrate	1.000
NH <sub>4</sub> Cl <sub>2</sub>	0.500
L-Tryptophan	2.000
KH <sub>2</sub> PO <sub>4</sub> (anhydrous)	9.520
Na <sub>2</sub> SO <sub>4</sub>	0.142
NaNO <sub>3</sub>	0.085
KCl	0.075
MgCl.6H <sub>2</sub> O	2.460
FeCl <sub>3</sub> .6H <sub>2</sub> O	1.08mg
MnCl <sub>2</sub> .4H <sub>2</sub> O	0.079
CaCl <sub>2</sub>	0.111
Water deionised	to 1000mL
pH	6.5

### *Streptomyces adenosporus* Medium

The medium was prepared according to Nishino & Murao (1974). 4% polypeptone and 2% glycerol were prepared in separate deionised Erlenmyer flasks (500mL) and the pH was adjusted to 7.0 with 1M KOH. The flasks were autoclaved at 121°C, 15psi for 15 minutes. 0.1%  $\text{KH}_2\text{PO}_4$ ; 0.04%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 2ppm  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  and 2ppm  $\text{MnSO}_4 \cdot 6\text{H}_2\text{O}$  were dissolved in order and pH was adjusted to 10.0 with 1M KOH and filter sterilised through a sterile 0.22 $\mu\text{m}$  nitrocellulose filter (Whatman). This solution was aseptically added to the aliquots of 4% polypeptone and 2% glycerol. Aliquots (25mL) of prepared medium (Table 6) were aseptically dispensed into sterile deionised Erlenmyer flasks (100mL).

## NUTRIENT LIMITATION EXPERIMENTS

### Inoculation

#### *Control Cultures*

Sterile L&C CDM or M9 media (25mL) in Erlenmyer flasks (100mL), were inoculated with two loopfuls of pure culture of either *B. subtilis* or *E. coli* respectively, from nutrient agar slopes. These were incubated at 37°C in an orbital incubator (200 osc min<sup>-1</sup>, Gallenkamp INA-305), overnight.

#### *Nutrient Limited Experimental Cultures.*

Sterile L&C CDM or M9 media (Tables 4 & 5) were prepared by the addition of various controlled concentrations of nutrients to be limited. Aliquots (25mL) of these media were dispensed into sterile deionised Erlenmyer flasks (100mL) and inoculated with 250 $\mu\text{L}$  of an overnight control culture of *B. subtilis* or *E. coli*. Cultures were then incubated at 37°C in an orbital incubator (200 osc min<sup>-1</sup>, Gallenkamp INA-305).

**Table 4:** Composition of nutrients limited within M9 medium  
(Koch & Gross 1979).

Component	Concentration
Carbon	$2.56 \times 10^{-2} \text{M}$ to $2.22 \times 10^{-6} \text{M}$
Nitrogen	$1.87 \times 10^{-3} \text{M}$ to $1 \times 10^{-5} \text{M}$
Magnesium	$1.0 \times 10^{-3} \text{M}$ to $1.0 \times 10^{-6} \text{M}$
Phosphate	$6.64 \times 10^{-2} \text{M}$ to $6.4 \times 10^{-7} \text{M}$

**Table 5:** Composition of nutrient limited within Leitch & Collier CDM  
(Leitch & Collier 1996).

Component	Concentration
Glucose	$2.56 \times 10^{-2} \text{M}$ to $1.5 \times 10^{-2} \text{M}$
Sodium citrate	$2.56 \times 10^{-2} \text{M}$ to $2.39 \times 10^{-2} \text{M}$
Glucose & Sodium citrate combined	$2.56 \times 10^{-2} \text{M}$ to $1.28 \times 10^{-2} \text{M}$
Magnesium	$1.21 \times 10^{-2} \text{M}$ to $1.21 \times 10^{-4} \text{M}$
Nitrogen	$1.9 \times 10^{-2} \text{M}$ to $9.355 \times 10^{-3} \text{M}$
Phosphate	$6.64 \times 10^{-2} \text{M}$ to $9.9 \times 10^{-3} \text{M}$
pH	6.5

## Enzyme Reaction Culture

### *Streptomyces adrophospholyticus* A-4668 NCIMB 13429 Culture

Aliquots (25mL) of the prepared medium (Table 6) in sterile, deionised Erlenmyer flasks (100mL) were inoculated with two loopfuls of *S. adrophospholyticus* and incubated aerobically (200 osc min<sup>-1</sup>, Gallenkamp INA-305) for 30 to 40 hours at 30°C (Nishino & Murao 1974).

**Table 6:** Composition of *S. adrophospholyticus* A-4668 medium (Nishino & Murao 1974)

Component	Concentration
Polypeptone	4%
Glycerol	2%
KH <sub>2</sub> PO <sub>4</sub>	0.1%
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.04%
FeSO <sub>4</sub> ·7H <sub>2</sub> O	2ppm
MnSO <sub>4</sub> ·6H <sub>2</sub> O	2ppm
pH	7.0

## Spectrophotometric Growth Assay

Sterile L&C CDM or M9 media (25mL) in Erlenmyer flasks (100mL), were inoculated with 250µL of an overnight control culture of either *B. subtilis* or *E. coli* respectively. Cultures were incubated according to the protocols in (page 59). Aliquots (2mL) were aseptically removed from control cultures at hourly intervals from zero time. These samples were observed at E470nm using a Cecil CE Series 2 Spectrophotometer blanked against a 2mL sample of sterile medium in a 1cm path length cuvette. The samples

were aseptically returned to their respective flasks after observation in order to ensure no alteration in the surface : volume ratio and hence, in overall aeration of the cultures. The absorbance at E470nm was plotted against time (Figs 5 & 6). This technique was repeated until stationary phase was observed. Experimental samples with an E470nm greater than 0.3 were diluted ten fold, and the true E470nm values were calculated (Lawrence & Maier 1977). This prevented deviations from linearity in optical densities above this value.

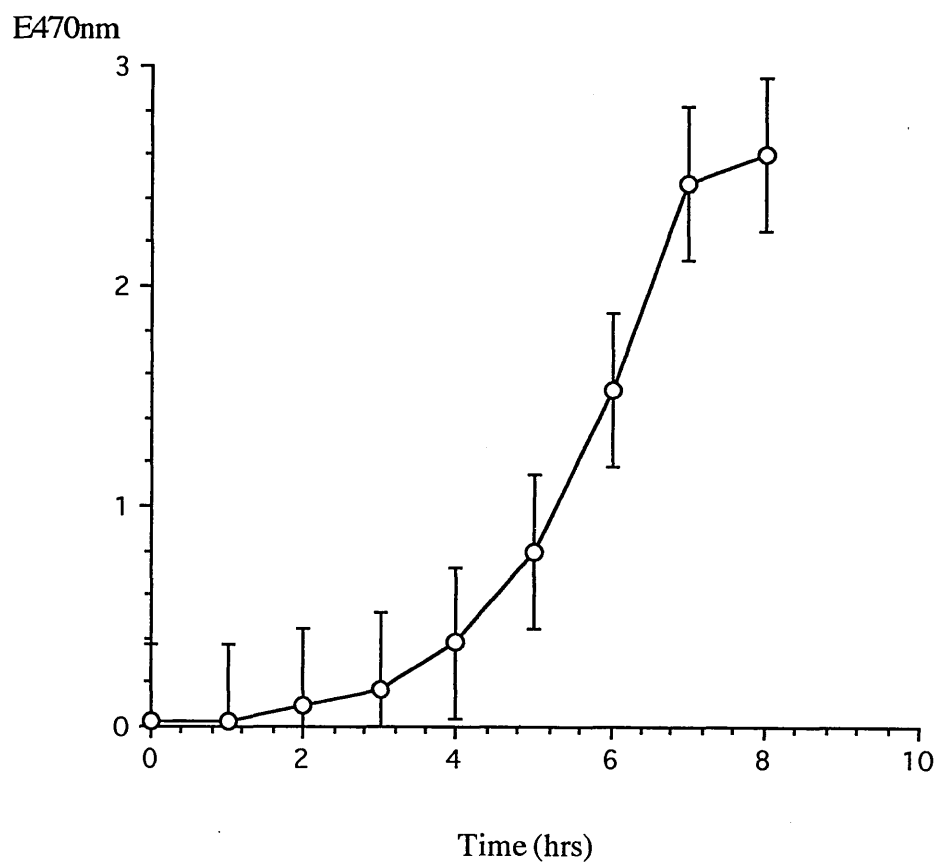
### *Viable Cell Number*

An aliquot (100 $\mu$ L) of bacterial cell suspension was aseptically removed from the test culture and placed in 9.9mL of sterile normal saline (0.9% w/v aq) and used for the preparation of serial dilutions in the range  $1 \times 10^{-2}$  to  $1 \times 10^{-16}$ . The diluted suspension was mixed thoroughly using a rotary vortex (Rotamixer 7930). Aliquots (100 $\mu$ L) of diluted cell suspension were spread onto the surfaces of sterile nutrient agar plates (Oxoid CM3), using a sterile glass spreader which had been flamed in alcohol and allowed to cool. This was performed in triplicate with appropriate dilutions from the range of sterile dilutions prepared. The inoculated nutrient agar plates were then incubated for 24 hours at 37°C. After incubation the colony forming units (CFU's) on the agar plates were counted. The number of CFU's per mL of original suspension was calculated by the use of the mathematical formula (Equation 3), where CFU mL<sup>-1</sup> is the number of colony forming units per mL of the original suspension, 1/DF is the reciprocal of the dilution factor and N is the number of colonies counted on the incubated agar plates.

$$\text{(Equation 3)} \quad \text{CFU mL}^{-1} = N (1/\text{DF})$$

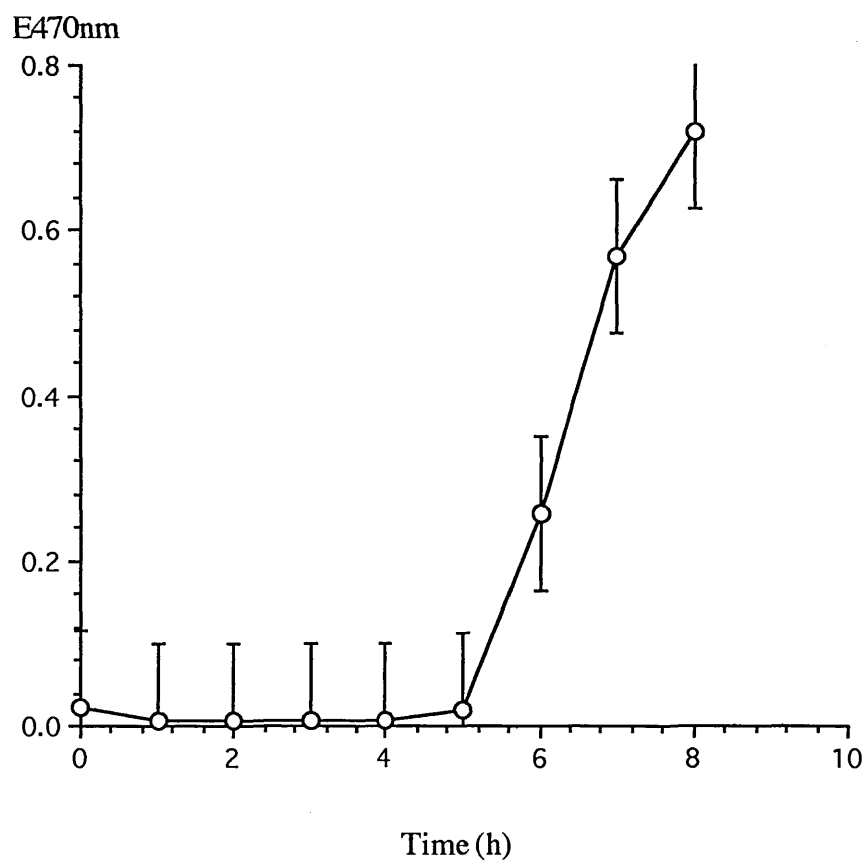
The numbers of bacterial cells present in the sample were also estimated using spectrophotometry. Subsequently, calibration curves were constructed (Figs 7 & 8) showing E470nm of a cell suspension and viable count against time. This was used in future experiments to determine cell concentrations as CFU mL<sup>-1</sup> at specific given E470nm values. This experiment was repeated for both *E. coli* and *B. subtilis*.

**Figure 5:** Growth (E470nm) of (o) *Escherichia coli* NCIMB 10000 in M9 medium against time. Error bars are calculated from and plotted as the Standard Error of the data set.

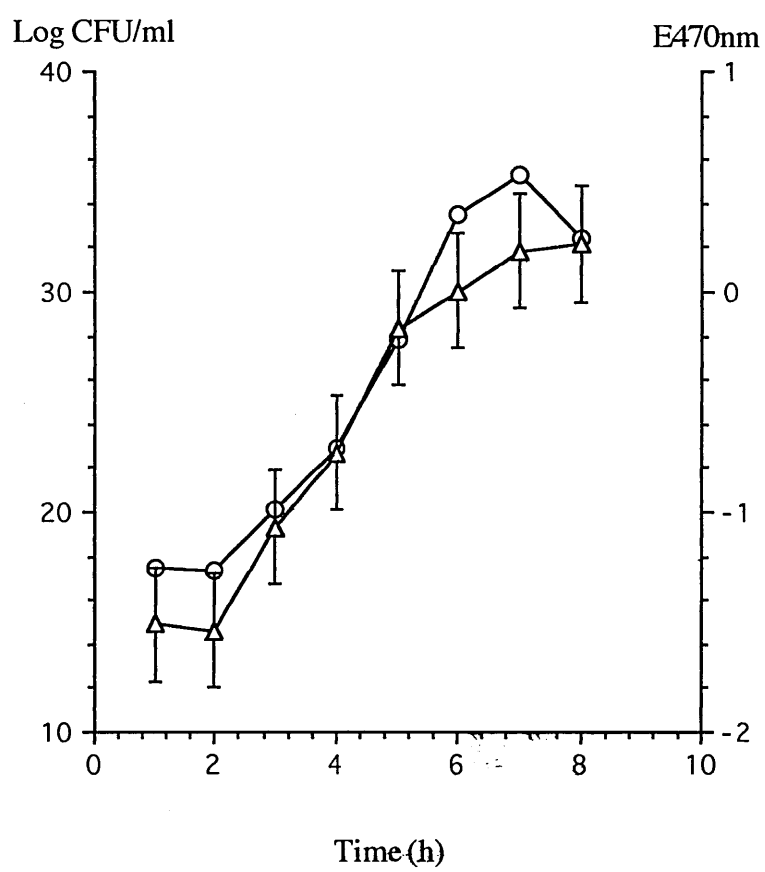




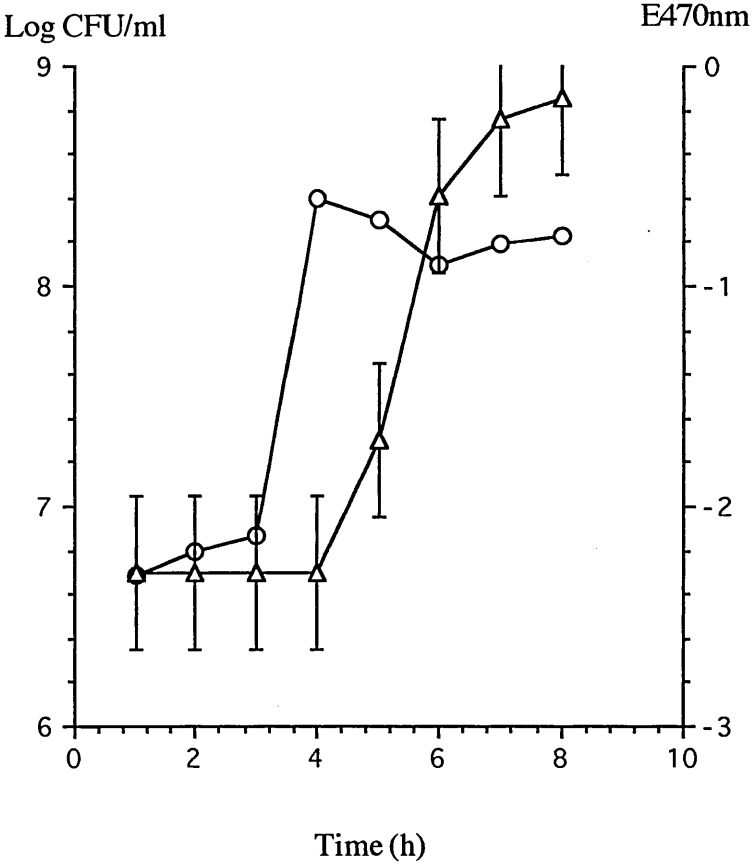
**Figure 6:** Growth (E470nm) of (o) *Bacillus subtilis* NCIMB 12900 in L&C CDM against time. Error bars are calculated from and plotted as the Standard Error of the data set.



**Figure 7:** Calibration curve of optical density (E470nm) (o) of *Escherichia coli* NCIMB 10000 against ( $\Delta$ ) cell density, as a function of time, facilitating the direct comparison of cell density and optical density. Error bars are calculated from and plotted as the Standard Error of the data set.



**Figure 8:** Calibration curve of optical density (E470nm) (o) of *Bacillus subtilis* NCIMB 12900 against ( $\Delta$ ) cell density, as a function of time, facilitating the direct comparison of cell density and optical density. Error bars are calculated from and plotted as the Standard Error of the data set.



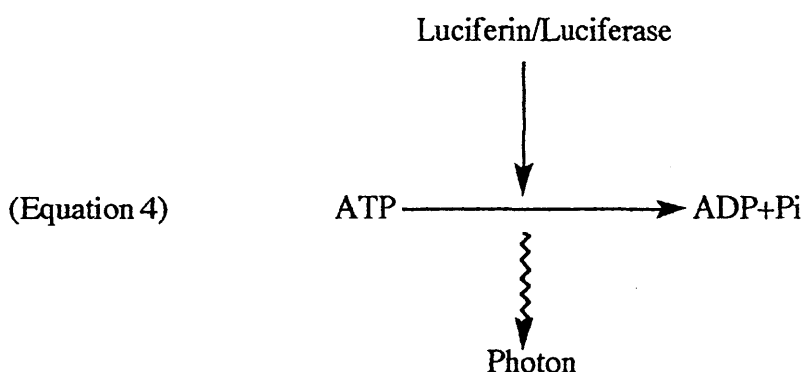
### *Total Cell Number*

Total cell enumeration was carried out using undiluted samples of the control cultures using haemocytometry (Improved Neubauer Haemocytometer). This was viewed microscopically using x40 objective magnification (Kyowa 765917, Tokyo) (Tortora *et al* 1992).

### **Assay of ATP**

#### *Intracellular Analysis of ATP*

Intracellular ATP levels were assayed *via* the luciferin/luciferase firefly extract system (Sigma, Equation 4, Wishart 1984).



Light production was monitored using a luminometer (Bio Orbit 1250) which was linked to a potentiometric chart recorder (Servogor 120 Chart Recorder, BBC Goerz Metrawatt, Austria). This enabled the observation and quantification of ATP present in the sample as a function of light released on reaction with luciferin/luciferase firefly extract. The intensity of light emission in this system is proportional to the concentration of ATP present.

#### *Firefly Extract Preparation*

An aliquot (5mL) of deionised water was added to a vial of luciferin/luciferase firefly stock extract. This was then diluted by the addition of 0.3mL of the stock firefly extract to 4.7mL of deionised water. An aliquot (800 $\mu$ L) of diluted extract was then drawn into a 1mL capacity syringe and this was placed securely into the rubber septum on top of the luminometer turret in preparation for application to luminometer tubes by depressing the syringe.

### *ATP Standard Preparation*

An aliquot (10 $\mu$ L) of 10 $\mu$ M ATP was added to 190 $\mu$ L of deionised water in a perspex luminometer tube. This was then placed inside the luminometer. The chart recorder was switched on and a base line was recorded. An aliquot of diluted firefly extract (800 $\mu$ L) was subsequently added to the ATP standard inside the luminometer chamber.

### *Sample Preparation*

An aliquot (200 $\mu$ L) of deionised water was heated in a pyrex tube in a Gallenkamp water bath. An aliquot (200 $\mu$ L) of bacterial cell suspension was removed every hour from the bacterial culture. The aliquot was added to a pyrex tube containing the 200 $\mu$ L of preheated water and boiled for 3 minutes to lyse the cells. The tube containing the lysed bacterial suspension was removed from the water bath after three minutes and cooled on ice. The sample was then centrifuged in a Sanyo MSE Microcentaur Microfuge (Sanyo, UK) for 1 minute at 11500xg, in order to remove the cell debris. An aliquot (200 $\mu$ L) of cell-free extract (supernatant) was placed in a perspex luminometer tube and the luminometer turret was moved to the 'sample in' position. The sample was then assayed for ATP by the addition of 800 $\mu$ L of dilute firefly extract into the luminometer turret.

## **Cellular Nucleotide Extraction**

### *Cellular Nucleotide Extraction of Bacterial Cultures.*

Aliquots (2 x 5mL) of bacterial overnight cultures were collected and treated with the addition of 0.5mL of 1.8% w/v formaldehyde. Samples were stored on ice for 25mins, they were centrifuged at 2325xg for 10mins. The supernatant was carefully decanted and discarded and the cell pellet was resuspended in 0.25mL of 0.1M potassium hydroxide and stored at 0°C for 30mins in order to lyse the cells. The cell suspension was neutralised by the addition of 2.5 $\mu$ L of 88% w/v phosphoric acid and centrifuged at 11500xg for 1.5hrs, in a cold room (4°C) to remove cell debris. The supernatant was removed and 0.5mL of 7mM KH<sub>2</sub>PO<sub>4</sub> buffer was added to the supernatant. The sample was filtered using 0.22 $\mu$ m nitrocellulose filter (Whatman), (Baracchini *et al* 1988, Little & Bremer 1982) and were stored at -70°C until required for analysis using 2D-TLC and HPLC.



## NUCLEOTIDE SEPARATION

### Two Dimensional Thin Layer Chromatography (2D-TLC)

#### *Nucleotide Standards*

Aliquots (10 $\mu$ L) of nucleotide (either AMP, GMP, ADP, GDP, ATP or GTP at concentrations of 20mg mL<sup>-1</sup> in sterile deionised water) were spotted on to a prepared PEI-cellulose plate (Sigma), at the origin, 2cm in and 3cm up from the lower right hand corner of the plate. The plate was dried after each spotting under a current of cold air. The plate was developed according to the procedures of Randerath and Randerath (1964).

#### *First Dimension Development*

The plate was placed into a chromatography tank containing approximately 1cm depth of 0.2M LiCl, and was left in the solvent for 2 minutes or until the solvent reached a height of 0.7 to 1cm. The plate was transferred to a second tank containing 0.6M LiCl, and developed for 5 minutes. The plate was then developed with 1.0M LiCl for 15 minutes and the final stage in the first dimension development was in 2.0M LiCl until the solvent front marked 13cms from point of origin was reached. The plate was then dried under a warm current of air, from a hairdryer, and was examined in a darkroom under short wave UV light ( $\lambda$ 254nm) to resolve the nucleotides. The plate was gently agitated in 500mL anhydrous methanol, (anhydrous methanol was prepared by filtering methanol through Whatman filter paper containing anhydrous sodium sulphate). The plate was then dried under a warm current of air. This treatment removed the lithium salts which would otherwise interfere with the elution in the second dimension.

#### *Second Dimension Development*

A line was scratched 15cm above the nucleotides separated by the first dimension development. The plate was developed in 0.5M Formic acid - Sodium formate solvent for 30 seconds. It was then transferred to 2.0M Formic acid - Sodium formate solvent for 2 minutes. The final development was in 4.0M Formic acid - Sodium formate solvent until the solvent front reached the line scratched on the plate. The plate was finally dried in a current of warm air and examined under short-wave UV light ( $\lambda$ 254nm). Each nucleotide was developed separately by this technique, then the process was repeated with

all 6 nucleotides in an equimolar mixture. The separated nucleotides eluted on the plates were traced onto tracing paper (Fig 9) and the *R<sub>f</sub>* values were determined (Table 7).

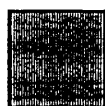
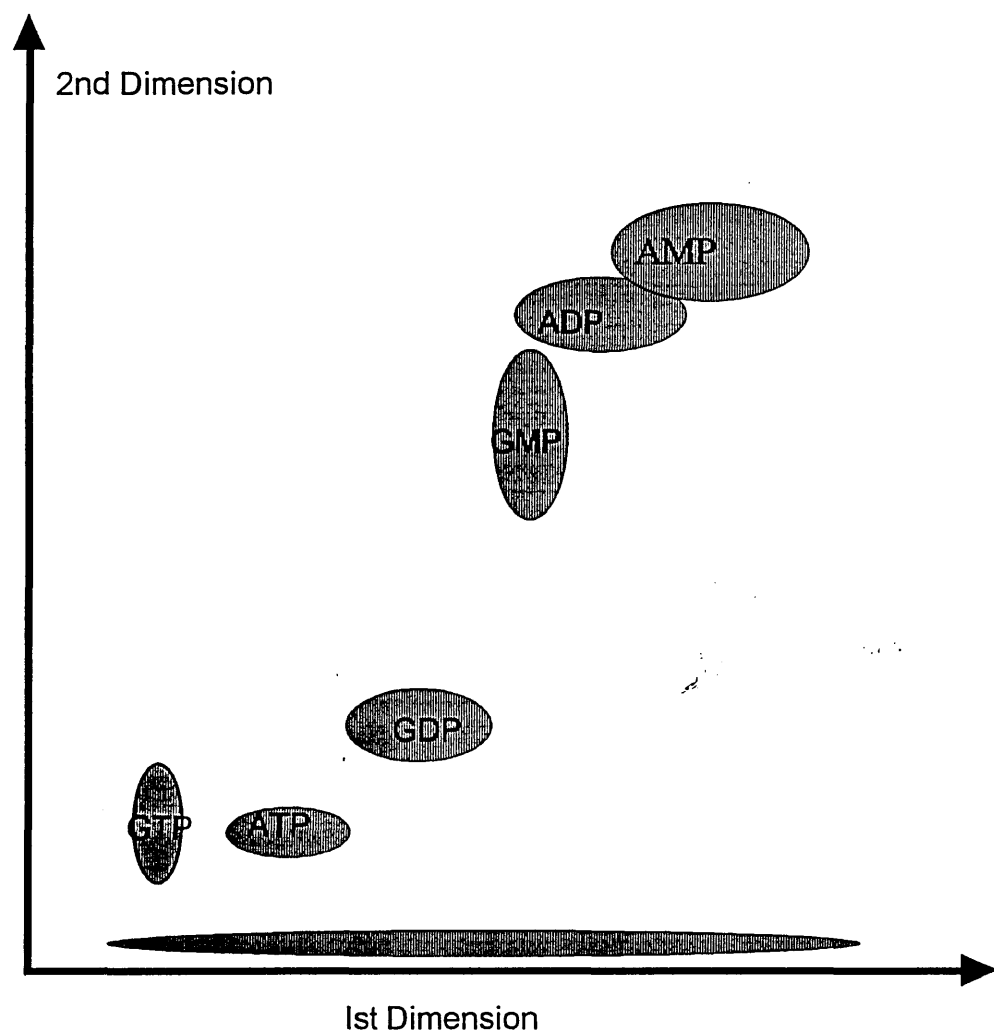
**Table 7:** *R<sub>f</sub>* values of a mixture of standard nucleotides chromatographed

Sample mixture of nucleotides	Dimension	<i>R<sub>f</sub></i> value (40mg mL <sup>-1</sup> )
Sample	1st	0.554*
AMP	2nd	0.6
GMP	“	0.51
ADP	“	0.463
GDP	“	0.1566
ATP	“	0.033
GTP	“	-

- = Not found

\* = *R<sub>f</sub>* value of combined spot containing AMP, GMP, ADP, GDP, ATP and GTP

**Figure 9:** 2D-TLC of 20  $\mu\text{L}$  of 40mg mL<sup>-1</sup> nucleotide standard controls  
(Randerath & Randerath 1964)



Nucleotide standards  
(20 mg/ml in 1M LiCl)

## High Performance Liquid Chromatography (HPLC)

### Standard Nucleotides

Aliquots (20 $\mu$ L) of nucleotides (either AMP, GMP, ADP, GDP, ATP or GTP at concentrations of  $1 \times 10^{-12}$  mg mL<sup>-1</sup> in sterile deionised water) were assayed using method of Ochi *et al* (1981) and Little and Bremer (1982). The nucleotides were eluted using a gradient buffer system over 65 minutes (Table 8) at a flow rate of 1.5mL per minute.

Buffer A: 7mM KH<sub>2</sub>PO<sub>4</sub> (pH4 adjusted with 1M H<sub>3</sub>PO<sub>4</sub>),

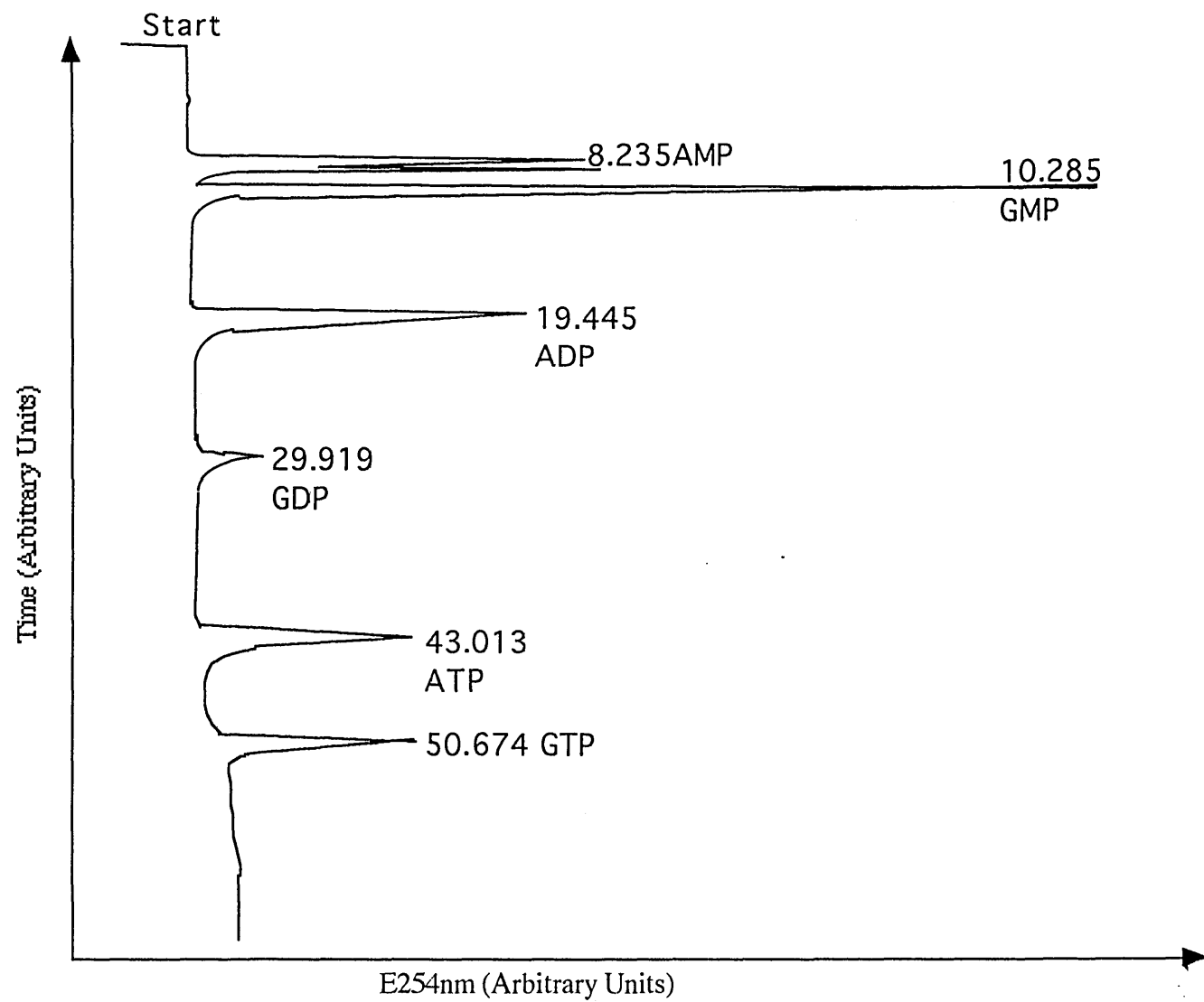
Buffer B: 0.5M KH<sub>2</sub>PO<sub>4</sub> + 0.5M Na<sub>2</sub>SO<sub>4</sub> (pH5 adjusted with 1M KOH).

The buffers were made up in sterilised dH<sub>2</sub>O, filtered using 0.22 $\mu$ m (Whatmans) nitrocellulose filter paper, and degassed for 30 minutes using a vacuum pump. Aliquots (20 $\mu$ L) of the nucleotide standards were loaded onto a Partisil PXS 10/25 SAX (Whatman), SO<sub>4</sub><sup>2-</sup> form column and were eluted using a Spectraphysics SP8800 Ternary HPLC Pump, Hewlett & Packard HP3396 Series 911 Integrator and Hewlett & Packard Series 1050 UV Detector. The retention time (R<sub>t</sub>) of each individual nucleotide was determined. The R<sub>t</sub> (Table 9) for a mixture of all 6 nucleotides in an equimolar concentration was also determined by the same techniques (Fig 10).

**Table 8:** HPLC Buffer Gradient Programme  
(Ochi *et al* 1981)

Time (mins)	%Buffer A	% Buffer B
0	100	0
20	80	20
40	53	47
45	0	100
65	0	100

**Figure 10:** HPLC trace of the mixture of nucleotide standards (10 mg mL<sup>-1</sup> each), eluted over a 65 minute gradient (Ochi *et al* 1981), flow rate 1.5mL min<sup>-1</sup>, with 7mM KH<sub>2</sub>PO<sub>4</sub> (pH4) and 0.5M Na SO<sub>4</sub> + 0.5M KH<sub>2</sub>PO<sub>4</sub> (pH5).



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**Table 9:** Retention times ( $R_t$ ) of nucleotide standards.

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Nucleotide 10mg mL <sup>-1</sup>	$R_t$ (mins)
AMP	8.235
GMP	10.285
ADP	19.445
GDP	29.919
ATP	43.013
GTP	50.674

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#### **Minimum Inhibitory Concentrations (MIC's)**

*Determination of Erythromycin, Ampicillin, Nalidixic Acid and Streptomycin MICs against E. coli and B. subtilis using the Tube Dilution Method.*

Culture tubes, each containing 9.0mL of sterile nutrient broth had aliquots of the antibiotic being tested added to give the following final antibiotic concentrations in a total volume of 10mL: 0, 2, 4, 8, 16, 32, 64, 128, 256 and 512 $\mu$ g mL<sup>-1</sup>. An aliquot (0.1mL) of a 25mL bacterial culture grown overnight in CDM in an orbital incubator (200osc/min, 37°C) was aseptically added to the previously prepared tubes. These tubes were then incubated statically at 37°C for 48 hours. During this period the tubes were examined for visible growth at 19 hours and 48 hours. The MIC was determined as the lowest concentration of antibiotic showing no visible growth after 19 hours. This experiment was repeated for both *E. coli* NCIMB 10000 and *B. subtilis* NCIMB 12900 for all four antibiotics (Table 10). Streptomycin was applied at a concentration range (0 to 20  $\mu$ g mL<sup>-1</sup> in 2  $\mu$ g mL<sup>-1</sup> intervals) and yielded no visible growth at any concentration. The serum concentrations were obtained from ABPI Data Sheet Compendium (Walker 1993) for comparison of levels used in the treatment of bacterial infections *in vivo*.



**Table 10:** Minimum Inhibitory Concentrations of *Escherichia coli* NCIMB 10000 and *Bacillus subtilis* NCIMB 12900 in Nutrient Broth.

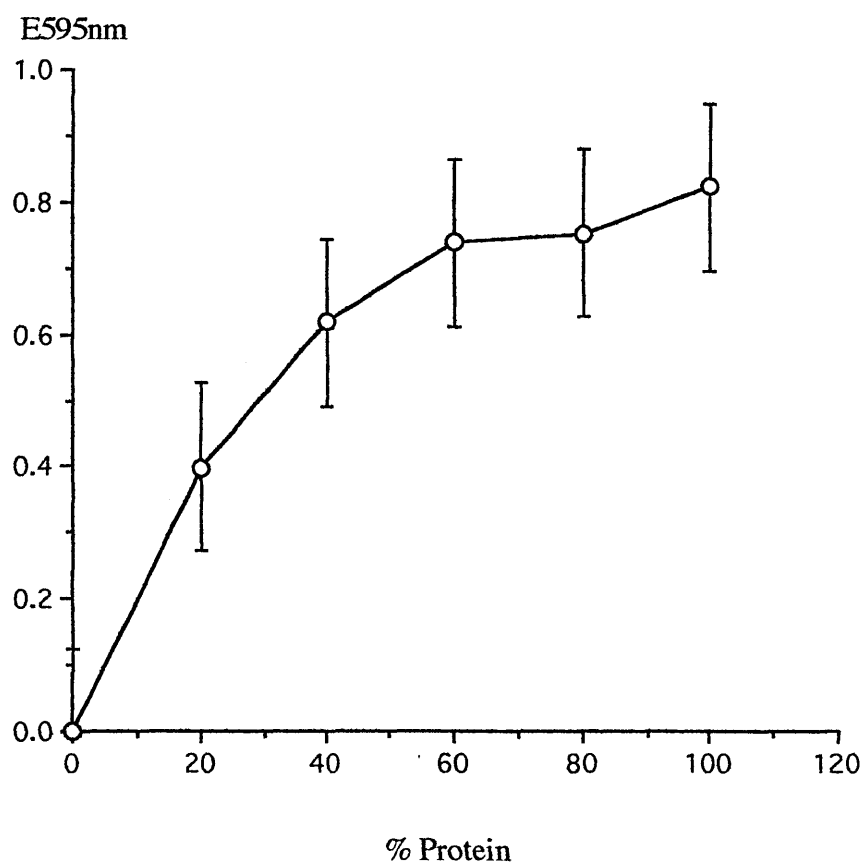
Antibiotic	<i>E. coli</i>	<i>B. subtilis</i>	Serum concentration (Walker 1993).
Ampicillin	5.0 $\mu\text{g mL}^{-1}$	125 $\mu\text{g mL}^{-1}$	35.7 to 143 $\mu\text{g mL}^{-1}$
Nalidixic acid	10 $\mu\text{g mL}^{-1}$	64 $\mu\text{g mL}^{-1}$	142 $\mu\text{g mL}^{-1}$
Streptomycin	N/A	15 $\mu\text{g mL}^{-1}$	143 $\mu\text{g mL}^{-1}$
Erythromycin	128 $\mu\text{g mL}^{-1}$	70 $\mu\text{g mL}^{-1}$	35.7 to 71.5 $\mu\text{g mL}^{-1}$
N/A - Not Achieved			

### Protein Analysis of HPLC Fraction

#### *Preparation of Protein Standard Calibration Curve* (Lowry *et al* 1951)

Six samples were prepared using the protein, bovine serum albumin (BSA) with protein concentrations ranging from 0% to 100% BSA. 100 $\mu\text{L}$  of BSA was added to 1mL of coomassie blue, incubated for 10 minutes at room temperature. The absorbency of the samples were observed spectrophotometrically at 595nm. The data obtained from these samples were used to construct a calibration curve (Fig 11). The HPLC fractions were analysed and absorbency was recorded at 595nm to determine protein content using the calibration curve. The HPLC fractions were also analysed spectrophotometrically at 280nm and 260nm (Adams *et al* 1992) to determine the nucleotide content of the sample by calculating the ratio between the two.

**Figure 11:** Comassie blue protein calibration curve concentrations of BSA (o) (0%, 20%, 40%, 60%, 80% and 100%) to determine the concentration of protein in HPLC fraction.



## **Statistical Methods**

All experiments in this project were designed to allow for statistical analysis and were performed in triplicate. Experimental data presented in this thesis represent the mean of those triplicate data sets. Where Standard Error bars are shown these were calculated *via* the methods of Hoel (1971).

## **EFFECTS OF NUTRIENT LIMITATION UPON CULTURES OF *Escherichia coli* NCIMB 10000 and *Bacillus subtilis* NCIMB 12900**

Under conditions where all the components required for energy transduction and formation of cell constituents are supplied, bacteria can grow and adapt to the ambient milieu. The growth of bacteria under poor conditions may produce resistant forms, such as spores, or they periodically enter and leave quiescent states, rather than reducing their growth rate (Koch 1997). When bacteria are forced to reduce their growth rate due to nutrient limitation they become more resistant to antimicrobial compounds. This increased susceptibility is due to the longer time available for drug penetration in the poorer medium (Koch & Gross 1979). Klemperer *et al* (1979) quantitatively determined the nutritional requirements for *E. coli* W3110 in batch culture for cells with (R<sup>+</sup>) and without (R<sup>-</sup>) the resistance plasmid RPI. The presence of the R plasmid affected the nutritional requirements of the cells. The RPI plasmid encodes for resistance to  $\beta$ -lactam antibiotics, kanamycin and tetracycline. The sensitivity of the cells containing the RPI plasmid to cetrимide, phenol and chlorohexidine was found to vary with the particular nutrient limited. For example the R<sup>+</sup> cells displayed a greater increase in resistance to cetrимide than did the R<sup>-</sup> cells and carbon limitation resulted in greater resistance than phosphate limitation. These nutrient limitation mediated changes in resistance may provide some insight into the mechanisms of drug exclusion by the cell envelope (Klemperer *et al* 1980).

Bacteria have a remarkable ability to adapt their metabolism in response to changing environmental conditions. The prime structure through which this adaptation is mediated is the bacterial cell envelope. The macromolecular structure and function of the cell envelope are largely determined by the growth environment and in particular, specific nutrient limitation, growth rate, growth temperature and replication in suspension or within a surface associated biofilm (Williams 1988). Non-growing stringent *Escherichia coli* cells under amino acid limitation produce peptidoglycan at a rate approximately 30% of that of growing cells (Nystrom & Kjelleberg 1989). The phenotypic changes which occur as the response to nutrient limitation are frequently associated with changes in resistance to

antimicrobial agents (Klemperer *et al* 1980).

In response to conditions of stress, bacteria are known to decrease the genetic expression of most normal cellular proteins and transiently over produce the so-called stress proteins (Neidhardt *et al* 1984). Starving bacteria are of interest in both an ecological and applied context, due to the physiological changes which occur during this process (Reeve *et al* 1984). Mutants of *E. coli* and *Salmonella typhimurium* that were unable to degrade proteins were found to have decreased stability under carbon starvation. Although these mutants had no genetic deficiency in their protein-synthetic machinery, they were unable to synthesise protein at the same rate as their corresponding wild-type during carbon starvation. This suggests that amino acids derived from protein degradation were utilised by these cells for new protein synthesis and that their increased susceptibility to carbon starvation originated from their inability to synthesis these proteins (Reeve *et al* 1984).

In *Bacillus subtilis*, nutrient limitations trigger the synthesis of numerous extracellular ( $\beta$ -glucanase) and intracellular enzymes, possibly in order to scavenge alternative nutrient sources. Many of the genes encoding these enzymes are subject to carbon-catabolite repression and are temporally activated when the cell enters the stationary phase after the exhaustion of readily metabolizable nutrients (Priest 1977, Fisher & Sonenshein 1991, Stulke *et al* 1993). Starving cells synthesise proteins whose purpose is to increase the probability of escaping the effects of starvation. Organisms subjected to carbon limitation depress the synthesis of catabolite enzymes thus, increasing their potential to make use of diverse carbon substrates and therefore, avoiding the effects of carbon limitation (Matin *et al* 1976, Matin 1979). A cell entering starvation faces a fundamentally altered physiological situation. The cell metabolism, previously geared to growth, must be reoriented to maintenance (Reeve *et al* 1984). Heterotrophic bacteria frequently experience a shortage of carbon and energy in oligotrophic marine waters (Nystrom & Kjelleberg 1989, Nystrom *et al* 1992, Kjelleberg *et al* 1993, Morita 1996). Carbon is an important factor for the successful development of the typical starvation-resistant cell seen in the marine *Vibrio* sp. strain S14 (Nystrom *et al* 1992, Holmquist & Kjelleberg 1993b). Bacteria under carbon limitation respond by producing molecules which possess regulatory functions. These regulatory molecules include the stationary phase sigma factor,  $\sigma$  (Hengge-Aronis 1993), and the ppGpp related mediators of the stringent response (Lazzarini *et al* 1971, Chaloner-Larsson & Yamazaki 1978, Xiao *et al* 1991, Spector & Cubitt 1992).

Sessile bacterial populations are metabolically quiescent, relative to their planktonic counterparts, as reflected by their slow growth rates (Gilbert *et al* 1987, La Tourette Prosser *et al* 1987, Murray *et al* 1987, Hoyle *et al* 1990). Within the biofilm glycocalyx, the sessile bacteria are shielded from the insults inflicted by surfactants (Govan 1975), antibodies (Baltimore & Mitchell 1980, Krieg *et al* 1988), and antibiotics (Nickel *et al* 1985, Brown *et al* 1988, Anwar *et al* 1989a, b, Hoyle & Costerton 1989).

It is necessary to understand the effects of single nutrient limitation upon bacterial cultures in order to determine the requirement ( $K_s$  value) the bacteria has for that particular nutrient. The  $K_s$  value will then indicate the limiting concentrations of specific nutrients used in future experimental work. Thus, enabling the observation of responses cultures of *E. coli* and *B. subtilis* exhibit when exposed to both specific nutrient limitation and antimicrobial agents.

### **Determination of $K_s$ values.**

#### *Preparation of M9 Medium*

Aliquots (100mL) of M9 salts (10-fold concentration) was prepared to contain  $\text{Na}_2\text{HPO}_4$ , 6g;  $\text{KH}_2\text{PO}_4$ , 3g;  $\text{NaCl}$ , 0.5g and  $\text{NH}_4\text{Cl}$ , 1g dissolved in order, in sterile  $\text{dH}_2\text{O}$ . This was dispensed into Erlenmyer flasks (200mL) and autoclaved at  $121^\circ\text{C}$ , 15psi for 15 minutes. Aliquots (50mL) of each of the following solutions were prepared in deionised water and dispensed into Erlenmyer flasks (100mL); 20% w/v glucose,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.1M) and  $\text{CaCl}_2$  (0.01M). These were autoclaved at  $121^\circ\text{C}$ , 15psi for 15 minutes. Aliquots (2.5mL) of M9 salts (10-fold concentration) were dispensed into sterile deionised Erlenmyer flasks (100mL). Aliquots (0.5mL) of 20% w/v glucose, (0.25mL)  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.1M), (0.25mL)  $\text{CaCl}_2$  (0.01M) and sterile  $\text{dH}_2\text{O}$  (21.5mL) were aseptically measured into the Erlenmyer flasks (100mL) containing the M9 salts. The flasks were inoculated with  $250\mu\text{L}$  of an overnight control culture of *E. coli*. Cultures were then incubated at  $37^\circ\text{C}$  in an orbital incubator (200 osc  $\text{min}^{-1}$ , Gallenkamp INA-305).

#### *Preparation of Carbon Limited M9 medium*

Aliquots (100mL) of M9 salts (10-fold concentration) were prepared as above. Aliquots (50mL) of each of the following solutions were prepared in deionised water and

dispensed into Erlenmyer flasks (100mL); 20% w/v glucose,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.1M) and  $\text{CaCl}_2$  (0.01M). The carbon source, glucose, was prepared using serial dilutions of the 20% w/v solution in sterile deionised water. The range of final carbon concentrations was  $2.22 \times 10^{-2}\text{M}$ ,  $1.11 \times 10^{-2}\text{M}$ ,  $5.55 \times 10^{-3}\text{M}$ ,  $2.22 \times 10^{-3}\text{M}$ ,  $2.22 \times 10^{-4}\text{M}$ ,  $2.22 \times 10^{-5}\text{M}$  and  $2.22 \times 10^{-6}\text{M}$ . These were autoclaved at  $121^\circ\text{C}$ , 15psi for 15 minutes. Aliquots (2.5mL) of M9 salts (10-fold concentration) were dispensed into sterile deionised Erlenmyer flasks (100mL). Aliquots of (0.25mL) of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.1M), (0.25mL) of  $\text{CaCl}_2$  (0.01M), (0.5mL) of the appropriate glucose solution and (21.5mL) of sterile  $\text{dH}_2\text{O}$  were aseptically added to the Erlenmyer flasks (100mL) containing M9 salts. The flasks of carbon limited M9 medium were inoculated and incubated as above. Results are given in figure 12.

#### *Preparation of Magnesium Limited M9 Medium.*

M9 salts (10-fold concentration) was prepared as above. Aliquots (50mL) of each of the following solutions were prepared in sterile  $\text{dH}_2\text{O}$  and dispensed into Erlenmyer flasks (100mL); 20% w/v glucose,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.1M) and  $\text{CaCl}_2$  (0.01M). The magnesium source,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , was prepared using serial dilutions of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.1M) solution in sterile  $\text{dH}_2\text{O}$ . The range of final  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  concentrations was  $1.0 \times 10^{-3}\text{M}$ ,  $5.0 \times 10^{-4}\text{M}$ ,  $1.0 \times 10^{-4}\text{M}$ ,  $5.0 \times 10^{-5}\text{M}$ ,  $1.0 \times 10^{-5}\text{M}$ ,  $5.0 \times 10^{-6}\text{M}$ ,  $1.0 \times 10^{-6}\text{M}$ ,  $1.0 \times 10^{-7}\text{M}$  and  $1.0 \times 10^{-8}\text{M}$ . These were autoclaved at  $121^\circ\text{C}$ , 15psi for 15 minutes. Aliquots (2.5mL) of M9 salts (10-fold concentration) were dispensed into sterile deionised Erlenmyer flasks (100mL). Aliquots of (0.5mL) of 20% w/v glucose, (0.25mL) of  $\text{CaCl}_2$  (0.01M), (0.25mL) of the appropriate  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  solution and sterile  $\text{dH}_2\text{O}$  (21.5mL) were aseptically added to the M9 salts. The flasks of magnesium limited M9 medium were inoculated and incubated as above. Results are given in figure 13.

#### *Preparation of Nitrogen Limited M9 Medium*

Aliquots (10mL) of M9 salts (10-fold concentration) were prepared  $\text{Na}_2\text{HPO}_4$ , 0.6g;  $\text{KH}_2\text{PO}_4$ , 0.3g and  $\text{NaCl}$ , 0.05g dissolved in sterile  $\text{dH}_2\text{O}$ , in order. To each appropriate flask  $\text{NH}_4\text{Cl}$  was added to give a final concentration of  $1.87 \times 10^{-2}\text{M}$ ,



$1.02 \times 10^{-2} \text{M}$ ,  $9.35 \times 10^{-3} \text{M}$ ,  $4.67 \times 10^{-3} \text{M}$ ,  $2.0 \times 10^{-3} \text{M}$ ,  $1.0 \times 10^{-3} \text{M}$ ,  $5.0 \times 10^{-4} \text{M}$ ,  $1.0 \times 10^{-4} \text{M}$  and  $1.0 \times 10^{-5} \text{M}$ . Sterile  $\text{dH}_2\text{O}$  was added to make up the volume to 10mL in Erlenmyer flasks (100mL) and autoclaved at  $121^\circ\text{C}$ , 15psi for 15 minutes. Aliquots (50mL) of each of the following solutions were prepared in deionised water and dispensed into the Erlenmyer flasks (100mL); 20% w/v glucose,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.1M) and  $\text{CaCl}_2$  (0.01M). The flasks of nitrogen limited M9 medium were inoculated and incubated as above. Results are given in figure 14.

#### *Preparation of Phosphate Limited M9 Medium*

Aliquots (10mL) of M9 salts (10-fold concentration) were prepared NaCl, 0.05g and  $\text{NH}_4\text{Cl}$ , 0.1g dissolved in order, in Tris-HCl buffer to maintain the buffered properties of the medium.

#### *Trizma HCl buffer*

Solution A: 0.2M Trizma base (24g Tris + 23.2g Maleic acid in 1 litre  $\text{dH}_2\text{O}$ ), solution B: 0.2N HCl. Trizma base-HCl buffer was prepared as follows: 25mL solution A + 25.5mL solution B made up to 100mL with sterile  $\text{dH}_2\text{O}$ .

To each appropriate flask  $\text{Na}_2\text{HPO}_4$  and  $\text{KH}_2\text{PO}_4$  were added to give a final concentration of  $6.4 \times 10^{-2} \text{M}$ ,  $3.2 \times 10^{-2} \text{M}$ ,  $6.4 \times 10^{-3} \text{M}$ ,  $6.4 \times 10^{-4} \text{M}$ ,  $6.4 \times 10^{-5} \text{M}$  and  $6.4 \times 10^{-6} \text{M}$ , there was a flask with medium which did not contain any added phosphate. Tris-buffer was added up to 10mL in the Erlenmyer flasks (100mL) and autoclaved at  $121^\circ\text{C}$ , 15psi for 15 minutes. Aliquots (50mL) of each of the following solutions were prepared in deionised water and dispensed into the Erlenmyer flasks (100mL); 20% w/v glucose,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.1M) and  $\text{CaCl}_2$  (0.01M). The flasks of phosphate limited M9 medium were inoculated and incubated as above. Results are given in figure 15.

#### *Leitch & Collier Chemically Defined Medium (L&C CDM)*

*Bacillus subtilis* 168 was originally grown in Dawes & Mandelstam (1970) sporulation medium. This medium did not provide sufficient cell densities for this experimental work and, hence, a new medium was developed, referred to hereafter as Leitch

and Collier medium (L&C CDM; Table 3) (Leitch & Collier 1996, Appendix 1). The concentrations of the four elements to be limited were increased to the same level as those in M9 medium (Koch & Gross 1979) used for growth of *E. coli*. The various media components for a single medium were dissolved in the given order in 800mL of sterile dH<sub>2</sub>O. The pH of the medium was then adjusted to pH 6.5 by the addition of 1M sodium hydroxide. The volume of the medium was adjusted to 1000mL. The medium was filter sterilised under vacuum through a sterile 0.22 $\mu$ m cellulose acetate membrane. After sterilisation aliquots (25mL) of the medium was aseptically dispensed into sterile deionised Erlenmyer flasks (100mL). The flasks were inoculated with 250 $\mu$ L of an overnight control culture of *B. subtilis*. Cultures were then incubated at 37°C in an orbital incubator (200 osc min<sup>-1</sup>, Gallenkamp INA-305). The new medium produced a higher cell density in a shorter period of time, thus enabling suitable nutrient limitations to be imposed upon the test microorganisms.

#### *Preparation of Carbon Limited L&C CDM*

The carbon source, glucose was limited. The L&C CDM was prepared with carbon concentrations of 2.56x10<sup>-2</sup>M, 2.41x10<sup>-2</sup>M, 2.28x10<sup>-2</sup>M, 2.0x10<sup>-2</sup>M, 1.87x10<sup>-2</sup>M, 1.72x10<sup>-2</sup>M and 1.45x10<sup>-2</sup>M. Results are given in figure 16.

#### *Preparation of Magnesium Limited L&C CDM*

The magnesium source, MgCl<sub>2</sub>·6H<sub>2</sub>O was limited. The L&C CDM was prepared with magnesium concentrations of 1.2x10<sup>-2</sup>M, 6.05x10<sup>-3</sup>M, 3.025x10<sup>-3</sup>M, 1.2x10<sup>-3</sup>M, 6.0x10<sup>-4</sup>M, 3.0x10<sup>-4</sup>M and 1.2x10<sup>-4</sup>M. Results are given in figure 17.

#### *Preparation of Nitrogen Limited L&C CDM*

The nitrogen source, L-Tryptophan was limited. The L&C CDM was prepared with nitrogen concentrations of 1.92x10<sup>-2</sup>M, 1.4x10<sup>-2</sup>M, 1.1x10<sup>-2</sup>M, 1.0x10<sup>-2</sup>M, 9.59x10<sup>-3</sup>M, 9.44x10<sup>-3</sup>M and 9.395x10<sup>-3</sup>M. Results are given in figure 18.

### *Preparation of Phosphate Limited L&C CDM*

The phosphate source,  $\text{KH}_2\text{PO}_4$  was limited. The L&C CDM was prepared with phosphate concentrations of  $6.99 \times 10^{-2}\text{M}$ ,  $5.24 \times 10^{-2}\text{M}$ ,  $3.056 \times 10^{-2}\text{M}$ ,  $2.939 \times 10^{-2}\text{M}$ ,  $2.2 \times 10^{-2}\text{M}$ ,  $1.469 \times 10^{-2}\text{M}$  and  $9.993 \times 10^{-3}\text{M}$ . Results are given in figure 19.

Aliquots (2mL) of the nutrient limited cultures *E. coli* and *B. subtilis* were aseptically removed at one hour intervals from zero time. These samples were observed at E470nm using a Cecil CE Series 2 Spectrophotometer blanked against a 2mL sample of sterile medium in a 1cm path length cuvette. The samples were aseptically returned to their respective flasks after observation in order to ensure no alteration in the surface : volume ratio and hence, in overall aeration of the cultures. This technique was repeated until stationary phase was observed. Experimental samples with an E470nm greater than 0.3 were diluted ten fold with sterile 0.9% saline, and the true E470nm values were calculated (Lawrence & Maier 1977). This prevented deviations from linearity in optical densities above this value. The absorbance at E470nm was plotted against time (Figs 12-19).

When compared to control cultures of both *E. coli* and *B. subtilis*, limitation of all four nutrients exhibited a reduction in growth rate of the cultures. The onset of stationary phase in comparison to the control cultures was affected by the limitation of certain nutrients but not in every case (Table 11). Carbon limitation exhibited the greatest effect upon *E. coli* cells. The cell density of *E. coli* was reduced by 86% and 39% in *B. subtilis*. The onset of stationary phase in *E. coli* was induced 2 hours earlier than in *B. subtilis* (Figs 12 & 16). The limitation of magnesium reduced the cell density of *E. coli* by 75% and 48% in *B. subtilis*. Magnesium limitation induced the onset of stationary phase in *E. coli* 1 hour earlier than controls and 2 hours earlier in *B. subtilis* (Figs 13 & 17). When exposed to nitrogen limitation the cell density of *E. coli* was reduced by 67% and 43% in *B. subtilis* cultures. The onset of stationary phase was 2 hours earlier in *E. coli* and 4 hours in *B. subtilis* (Figs 14 and 18) than in control cultures. Phosphate limitation exhibited a reduction of 50% of the cell density of *E. coli* and 29% in *B. subtilis*. The entry into stationary phase of *E. coli* was 1 hour earlier than controls and in *B. subtilis* cultures the onset of stationary phase was not affected when compared to control culture (Figs 15 and 19).

*B. subtilis* cells grow at a slower rate than *E. coli* cells. Therefore, *B. subtilis* cells are exposed to adverse conditions for longer periods of time than the *E. coli* cells and

have longer to attenuate themselves to these conditions. The slow rate of growth of *B. subtilis*

**Table 11:** Effects of nutrient limitation on growth parameters of *Escherichia coli* NCIMB 10000 and *Bacillus subtilis* NCIMB 12900

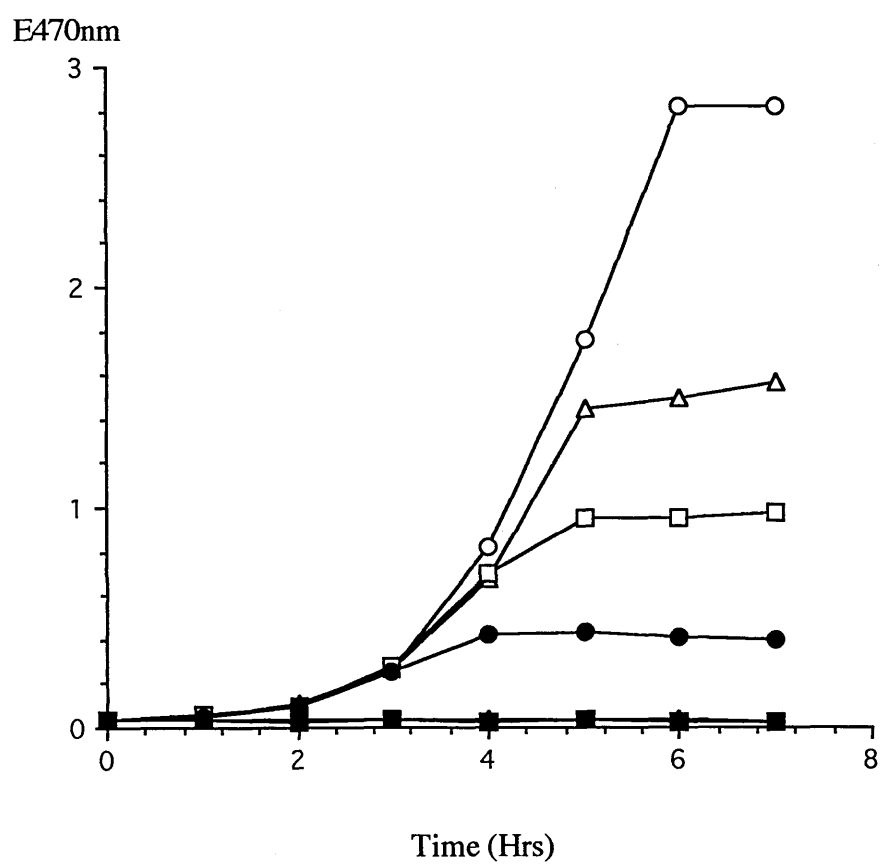
Nutrient	Minimum concentration at which growth rate changes were observed		Onset of stationary phase (hrs)		Cell density (% of Control)	
	<i>E.coli</i>	<i>B.subtilis</i>	<i>E.coli</i>	<i>B.subtilis</i>	<i>E. coli</i>	<i>B.subtilis</i>
Control	NA	NA	6/7	7	100%	100%
Carbon	2.22x10 <sup>-3</sup> M	1.45x10 <sup>-2</sup> M	3	5	86%	39%
Magnesium	5.0x10 <sup>-5</sup> M	3.025x10 <sup>-3</sup> M	5	7	75%	48%
Nitrogen	1.0x10 <sup>-3</sup> M	1.0x10 <sup>-2</sup> M	5	8	67%	43%
Phosphate	3.2x10 <sup>-2</sup> M	9.9x10 <sup>-3</sup> M	4	9	50%	29%

NA = Not applicable

may explain why the results of the nutrient limitation exposed *B. subtilis* cultures are not as well defined as those exhibited by *E. coli*. The L&C CDM contained two sources of carbon (glucose & sodium citrate) and nitrogen (ammonium chloride & L-tryptophan). Medium containing glucose as the sole source of carbon, resulted in low growth rates of *B. subtilis*, therefore a second carbon source was necessary. In order to achieve high growth rates only one source of carbon (glucose) was limited (Dawes & Mandelstam 1970). The bacteria may have utilised glucose first, and after it's exhaustion utilised sodium citrate in a process known as diauxic growth. This may explain why the observed growth of the *B. subtilis* under carbon limitation was not as obvious as the observed growth of *E. coli* under carbon

limitation. Nitrogen was limited by reducing the levels of L-tryptophan only as the concentration of ammonium chloride was already at the limiting concentration in M9 medium for *E. coli*. It is possible that L-tryptophan may be used for protein synthesis and does not contribute to the overall growth of the organism. Phosphate limitation did not appear to adversely affect the growth rate of *B. subtilis*. However, the growth rate of phosphate limited *E. coli* cultures was reduced when compared to that of the control culture. There was, however, a relatively high level of cell density observed in the culture incubated in medium with no added phosphate. The phosphate may be present solely to buffer the medium and does not contribute to growth requirements. It may also be possible for the bacteria to obtain phosphate from the other complex chemical constituents of the the medium thus, never truly being starved of phosphate. The apparent inability to induce classical nutrient limitation in *B. subtilis* may be due to the fact that the CDM used for this organism contained many more constituents of a chemically complex nature than the M9 medium used for *E. coli*. Therefore, the *B. subtilis* medium contained potentially higher concentrations of carbon and nitrogen from the other biochemically complex constituents of the medium. The cells may scavenge nutrients from these constituents and utilise them for growth.

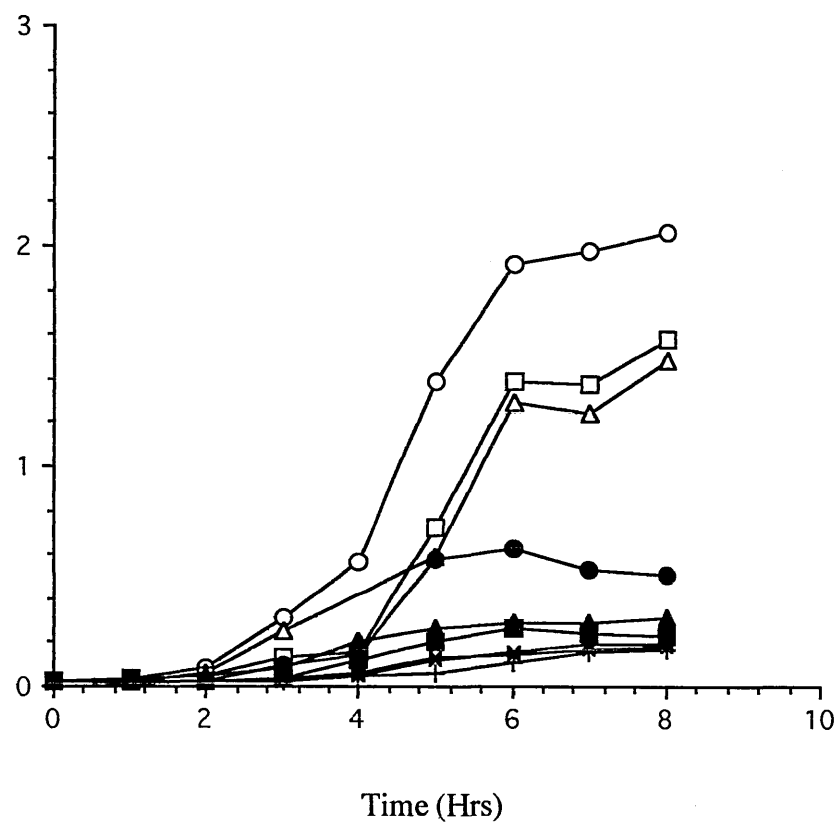
**Figure 12:** Growth (E470nm) of *Escherichia coli* NCIMB 10000 in carbon limited M9 medium. Carbon concentrations; (O)  $2.22 \times 10^{-2} \text{M}$ ; ( $\Delta$ )  $1.11 \times 10^{-2} \text{M}$ ; ( $\square$ )  $5.55 \times 10^{-3} \text{M}$ ; ( $\bullet$ )  $2.22 \times 10^{-3} \text{M}$ ; ( $\blacktriangle$ )  $2.22 \times 10^{-4} \text{M}$ ; ( $\blacksquare$ )  $2.22 \times 10^{-5} \text{M}$ ; ( $\times$ )  $2.22 \times 10^{-6} \text{M}$ . For reasons of clarity error bars have been deleted from this figure.



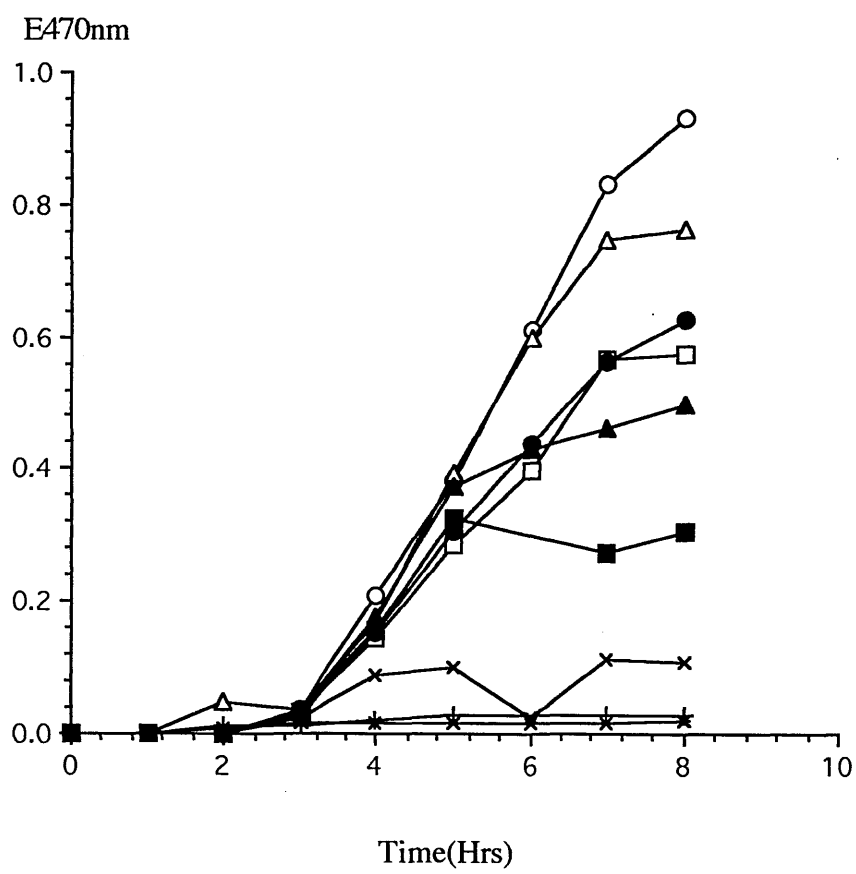
**Figure 13:** Growth (E470nm) of *Escherichia coli* NCIMB 10000 in magnesium limited M9 medium. Magnesium concentrations; (O)  $1.0 \times 10^{-3} \text{M}$ ; ( $\Delta$ )  $5 \times 10^{-4} \text{M}$ ; ( $\square$ )  $1.0 \times 10^{-4} \text{M}$ ; ( $\bullet$ )  $5 \times 10^{-5} \text{M}$ ; ( $\blacktriangle$ )  $1.0 \times 10^{-5} \text{M}$ ; ( $\blacksquare$ )  $5 \times 10^{-6} \text{M}$ ; ( $\times$ )  $1.0 \times 10^{-7} \text{M}$ . For reasons of clarity error bars have been deleted from this figure.



E470nm

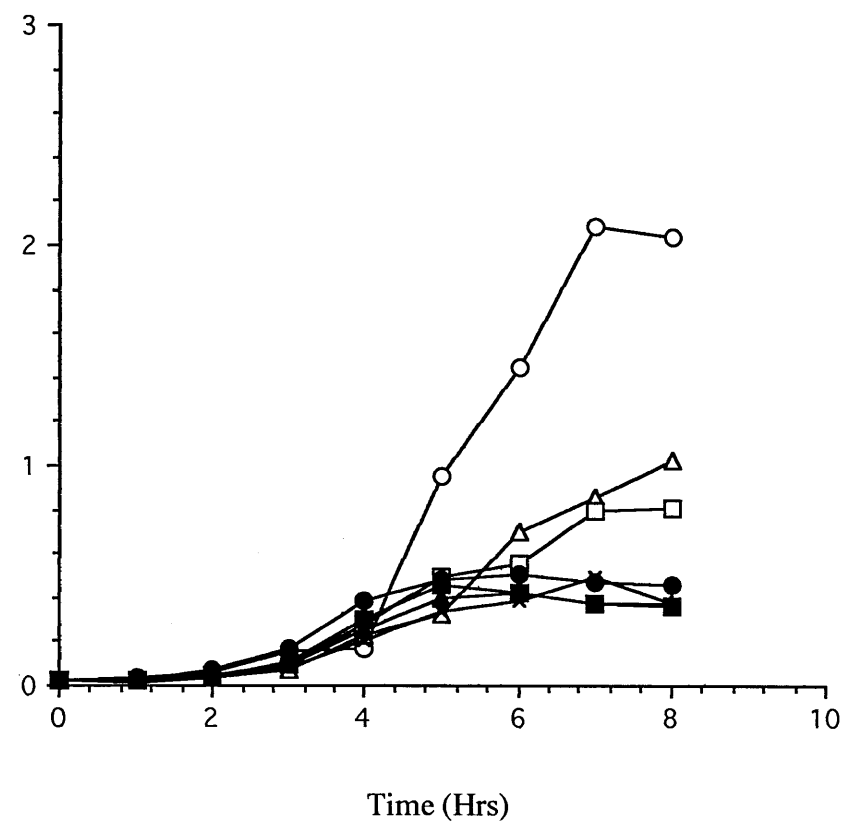


**Figure 14:** Growth (E470nm) of *Escherichia coli* NCIMB 10000 in nitrogen limited M9 medium. Nitrogen concentrations; (O)  $1.87 \times 10^{-2} \text{M}$ ; ( $\Delta$ )  $1.02 \times 10^{-2} \text{M}$ ; ( $\square$ )  $9.35 \times 10^{-3} \text{M}$ ; ( $\bullet$ )  $4.674 \times 10^{-3} \text{M}$ ; ( $\blacktriangle$ )  $2.0 \times 10^{-3} \text{M}$ ; ( $\blacksquare$ )  $1 \times 10^{-3} \text{M}$ ; ( $\times$ )  $5 \times 10^{-4} \text{M}$ ; (+)  $1 \times 10^{-4} \text{M}$ ; (\*)  $1 \times 10^{-5} \text{M}$ . For reasons of clarity error bars have been deleted from this figure.

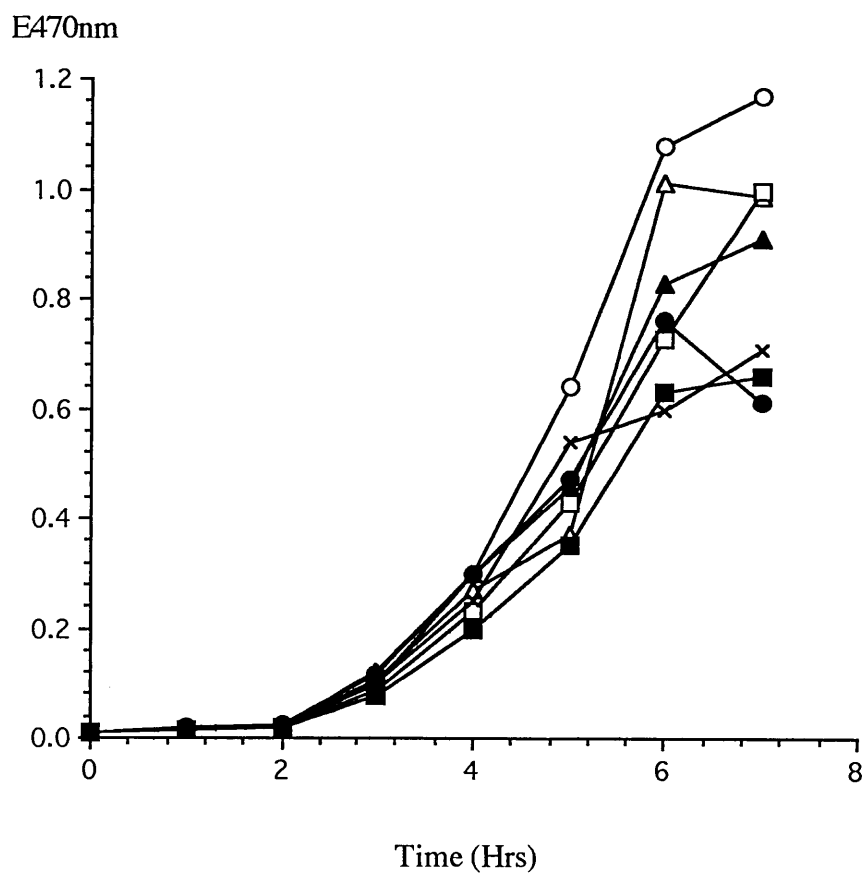


**Figure 15:** Growth (E470nm) of *Escherichia coli* NCIMB 10000 in phosphate limited M9 medium. Phosphate concentrations; (O)  $6.4 \times 10^{-2} \text{M}$ ; ( $\Delta$ )  $3.2 \times 10^{-2} \text{M}$ ; ( $\square$ )  $6.4 \times 10^{-3} \text{M}$ ; ( $\bullet$ )  $6.4 \times 10^{-4} \text{M}$ ; ( $\blacktriangle$ )  $6.4 \times 10^{-5} \text{M}$ ; ( $\blacksquare$ )  $6.4 \times 10^{-6} \text{M}$ ; ( $\times$ ) 0.0M. For reasons of clarity error bars have been deleted from this figure.

E470nm

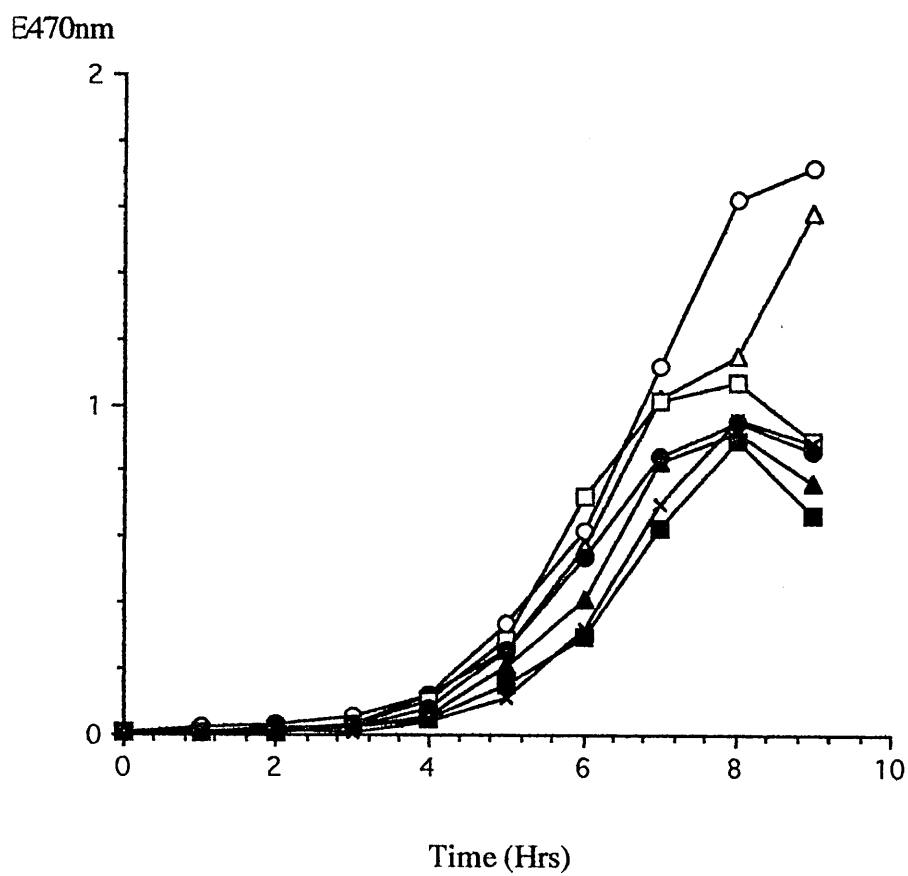


**Figure 16:** Growth (E470nm) of *Bacillus subtilis* NCIMB 12900 in carbon limited L&C CDM medium. Carbon concentrations; (O)  $2.56 \times 10^{-2} \text{M}$ ; ( $\Delta$ )  $2.41 \times 10^{-2} \text{M}$ ; ( $\square$ )  $2.28 \times 10^{-2} \text{M}$ ; ( $\bullet$ )  $2.0 \times 10^{-2} \text{M}$ ; ( $\blacktriangle$ )  $1.87 \times 10^{-2} \text{M}$ ; ( $\blacksquare$ )  $1.72 \times 10^{-2} \text{M}$ ; ( $\times$ )  $1.45 \times 10^{-2} \text{M}$ . For reasons of clarity error bars have been deleted from this figure.

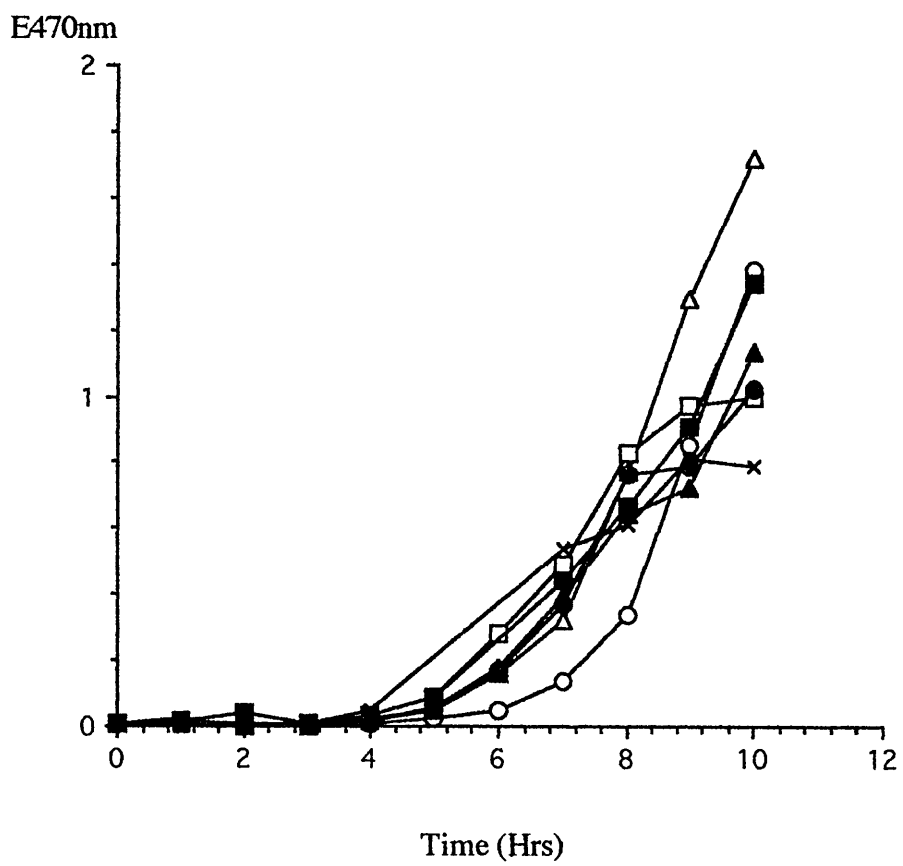


**Figure 17:** Growth (E470nm) of *Bacillus subtilis* NCIMB 12900 in magnesium limited L&C CDM medium. Magnesium concentrations; (O)  $1.2 \times 10^{-2} \text{M}$ ; ( $\Delta$ )  $6.05 \times 10^{-3} \text{M}$ ; ( $\square$ )  $3.025 \times 10^{-3} \text{M}$ ; ( $\bullet$ )  $1.2 \times 10^{-3} \text{M}$ ; ( $\blacktriangle$ )  $6.0 \times 10^{-4} \text{M}$ ; ( $\blacksquare$ )  $3.0 \times 10^{-4} \text{M}$ ; ( $\times$ )  $1.2 \times 10^{-4} \text{M}$ . For reasons of clarity error bars have been deleted from this figure.

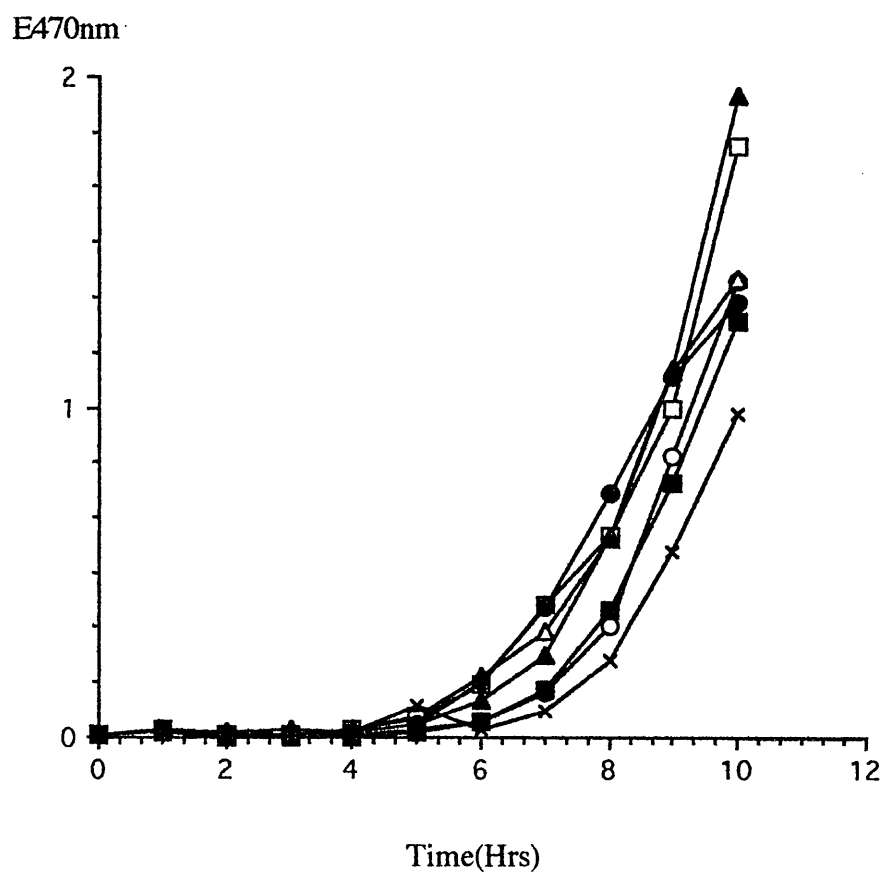




**Figure 18:** Growth (E470nm) of *Bacillus subtilis* NCIMB 12900 in nitrogen limited L&C CDM medium. Nitrogen concentrations; (O)  $1.9 \times 10^{-2} \text{M}$ ; ( $\Delta$ )  $1.4 \times 10^{-2} \text{M}$ ; ( $\square$ )  $1.1 \times 10^{-2} \text{M}$ ; ( $\bullet$ )  $1.0 \times 10^{-2} \text{M}$ ; ( $\blacktriangle$ )  $9.59 \times 10^{-3} \text{M}$ ; ( $\blacksquare$ )  $9.44 \times 10^{-3} \text{M}$ ; ( $\times$ )  $9.395 \times 10^{-3} \text{M}$ . For reasons of clarity error bars have been deleted from this figure.



**Figure 19:** Growth (E470nm) of *Bacillus subtilis* NCIMB 12900 in phosphate limited L&C CDM medium. Phosphate concentrations; (O)  $6.99 \times 10^{-2} \text{M}$ ; ( $\Delta$ )  $5.24 \times 10^{-2} \text{M}$ ; ( $\square$ )  $3.056 \times 10^{-2} \text{M}$ ; ( $\bullet$ )  $2.939 \times 10^{-2} \text{M}$ ; ( $\blacktriangle$ )  $2.2 \times 10^{-2} \text{M}$ ; ( $\blacksquare$ )  $1.469 \times 10^{-2} \text{M}$ ; ( $\times$ )  $9.993 \times 10^{-3} \text{M}$ . For reasons of clarity error bars have been deleted from this figure.



## **Growth rate**

Cultures exposed to nutrient limitation reduce their growth rate in order to fully utilise the nutrients available (Kjelleberg *et al* 1993). The immediate response of rapidly growing cells to nutrient limitation is a shutdown of RNA, protein synthesis and peptidoglycan synthesis (Kjelleberg *et al* 1993). The cell division processes of bacterial cultures under nutrient limited conditions are likely to be heavily influenced by the availability of critical nutrients. The burden of nutrient deprivation results in physiological changes in a number of ways; (i) The utilisation of nutrients is rationed within the cell by utilising alternative substrates, modification of the cell composition, and/or reduction in the amounts of cellular macromolecules containing such nutrients. (ii) The cell surface is adapted to increase the affinity of surface components for the growth-limiting substrate in order to ensure that uptake in the cytosol is more competitive. (iii) The cellular growth rate is reduced to give the maximum permissible (given i and ii) (Brown *et al* 1990). Growth limitation by different nutrients therefore, gives rise to cells with reduced growth rates and altered cell envelopes (Hansen *et al* 1975, Williams 1988, Brown *et al* 1990, Holmquist & Kjelleberg 1993b).

## **Protein synthesis**

The macromolecular composition of bacterial cells varies as a function of growth rate. In particular, the number of ribosomes is regulated such that, at any specific growth rate, it is just adequate to sustain the corresponding rate of protein synthesis (Hansen *et al* 1975). Flardh *et al* (1992) suggested that ribosomes were lost slowly during carbon starvation in *Vibrio* spp., and that, they existed in excess in comparison to the apparent demand for protein synthesis. Carbon starvation results in the synthesis of new proteins which are beneficial for survival during starvation (Groat & Matin 1986). The synthesis of protein during starvation is of paramount importance. The protein synthesis occurs in order to maintain the viability of the cells (Reeve *et al* 1984). The considerable cost to the cell in a nutrient deprived environment results in mechanisms which efficiently economise protein synthesis in the complete absence of exogenous substrates. The two main strategies are either to synthesise large amounts of mRNA, or to synthesis mRNA which is stable enough to translate many proteins from one message (Albertson *et al* 1990).

The results obtained from the nutrient limitation studies enabled the

determination of the bacteria's requirement for each nutrient. Lineweaver-Burk plots (Lineweaver & Burk 1934) of the reciprocal of rate of growth ( $1/V$ ) against the reciprocal of the substrate concentration ( $1/[S]$ ) were created with the data obtained from the nutrient limitation experiments a typical plot is given in Figure 20. The  $K_S$  (Monod constant) of the cultures was calculated from these graphs (Table 12).

**Table 12:** Table of  $K_S$  values for various substrates against *E. coli* and *B. subtilis* as determined via the growth rate effect method

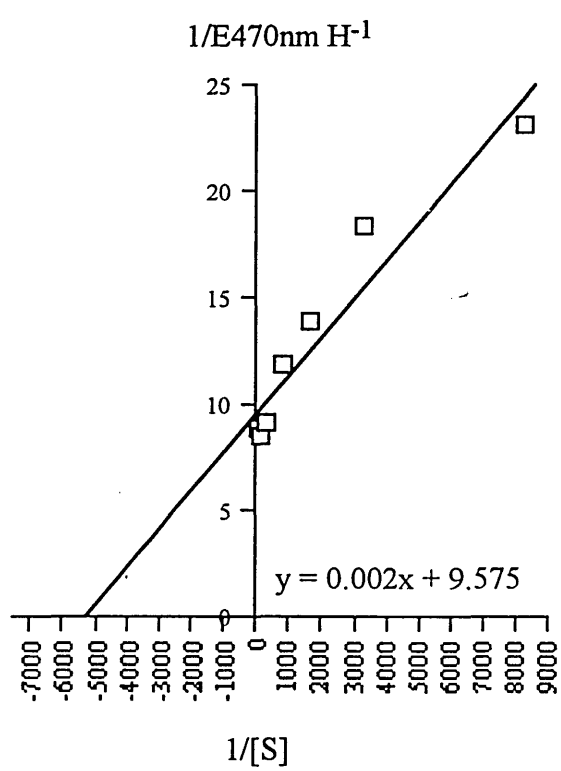
Substrate	$K_S$ ( $\mu\text{M}$ )	
	<i>Escherichiacoli</i>	<i>Bacillus subtilis</i>
Carbon	500.00	250,000.00
Nitrogen	125.00	4.54
Magnesium	35.70	185.00
Phosphorous	42.55	2660.00

In general, the lower the  $K_S$  value the greater the requirement that organism has for that specific nutrient. From the results of the  $K_S$  studies (Table 12), it is clear that *E. coli* has a greater requirement for the limited nutrients than *B. subtilis*. This was reflected in the limitation results. The only nutrient which *B. subtilis* had a greater requirement for than *E. coli* was nitrogen. The nitrogen source for *B. subtilis* was the amino acid L- tryptophan and it is possible that this may be used for protein synthesis and was not utilised as a growth constituent.

Monod (1949) suggested that nutrient limitation can indicate total starvation for one or more essential nutrients. Chronic starvation occurs when nutrients are approaching the lowest level required for the cellular uptake systems to function. These low levels of

**Figure 20:** A typical Lineweaver-Burk plot of magnesium limitation of *Bacillus subtilis* NCIMB 12900.





nutrients result in frequent periods of total nutrient starvation. Chronic starvation allows the culture to grow at a slower rate enabling the maximum growth potential of the culture under given environmental conditions. Chronic starvation can also provoke the synthesis of inducible enzymes and transport systems in order to overcome the nutritional stress by way of shifting to more energy-productive catabolic pathways. Low growth rates can lead to protein mistranslation, which may have increased occurrence during the growth of the bacterial cells (Gallant & Foley 1979). In order to prevent these errors occurring cells undertake the production of a major nucleotide effector guanosine-3'-diphosphate-5'-diphosphate (ppGpp, Magic Spot I, MSI). This highly phosphorylated nucleotide (HPN) is produced during stringent response. It amplifies the proofreading of protein synthesis under low growth rates. ppGpp also regulates the transcription of specific genetic units as well as regulating some key enzymes in various metabolic pathways during nutritional stress (Hara & Sy 1983). This response is similar to the sporulation of differentiating bacteria found in ecosystems which are subjected to environmental stresses.

During the onset of stationary phase bacterial cells produce two HPN's, guanosine-3'-diphosphate-5'-diphosphate (ppGpp, Magic Spot I, MSI) and guanosine-3'-diphosphate-5'-triphosphate (pppGpp, Magic Spot II, MSII). The production of these HPN's aids the survival of the bacterial cells under environmental stresses such as nutrient limitation (Cashel & Gallant 1969, Block & Haseltine 1974, Chaloner-Larsson & Yamazaki 1975, Gallant & Lazzarini 1976, Cashel & Rudd 1987, O'Farrell 1978, Lagosky & Chang 1980, Fehr & Richter 1981(a), Kelly *et al* 1991, Gustafson *et al* 1993, Kjelleberg *et al* 1993). During the onset of stationary phase the cells also produce secondary metabolites, particularly antibiotics (Schaeffer 1969, Katz & Demain 1977, Ochi and Ohsawa 1984, Ochi 1987, Zuber *et al* 1993). It has been suggested that the production of these antibiotics also enhance the survival of the bacteria when they are in a dormant state (Bu'lock 1961, Schaeffer 1969, Katz & Demain 1977, Bennett & Bentley 1989, Nakano & Zuber 1990, Vinning 1990). During slow growth rate (Schaechter *et al* 1958, Koch & Gross 1979, Tempest *et al* 1983, Kjelleberg *et al* 1987, Brown *et al* 1990, Kjellberg *et al* 1993, Koch 1997), dormancy (Novitsky & Morita 1977, Dow *et al* 1983, Nystrom & Kjelleberg 1989, Gilbert *et al* 1990), stringent response (SR) (Cashel & Gallant 1969, Freese & Heinze 1984, Cashel & Rudd 1987) and sporulation (Flardh & Kjelleberg 1994, Nystrom *et al* 1990, Nystrom *et al* 1992) bacteria develop resistance to environmental insults and the future

progeny of these “*resting cultures*” retain these resistant properties (Russell & Chopra 1990). As the onset of stationary phase of the bacterial growth produces these elegant survival mechanisms it is hypothesised that there may be a direct link between the production of the HPN’s and resistance to antibiotics.

**PROCEDURES FOR THE ISOLATION AND  
IDENTIFICATION OF NUCLEOTIDES IN CELLULAR  
EXTRACTS OF *Escherichia coli* NCIMB 10000 and *Bacillus  
subtilis* NCIMB 12900**

The production of 'Magic Spot' during the growth of *Bacillus* spp. and *E. coli* under conditions of nutrient limitation has been investigated in depth (Cashel & Gallant 1969, Hamel & Cashel 1973, Cashel 1975, Chaloner-Larsson & Yamazaki 1975, Rhaese *et al* 1976, Van Ooyen *et al* 1976, Murao *et al* 1980, Pun & Pennington 1980, Nishino & Murao 1981, Hara & Sy 1983, Moat & Foster 1988, Rojas & Ehrenberg 1991, Mukai 1992). For the purposes of this project it was imperative that a rapid detection method was employed in order to determine the presence of HPNs in bacterial cells. A literature search revealed that numerous techniques had been utilised in order to identify the production of HPNs in bacteria exposed to nutrient limitation or starvation. These techniques include Thin Layer Chromatography (TLC; Bochner & Ames 1982a), Two Dimensional Thin Layer Chromatography (2-D TLC; Randerath & Randerath 1964, Lagosky & Chang 1980, Bochner & Ames 1982b) and High Performance Liquid Chromatography (HPLC; Little & Bremer 1982, Payne & Ames 1982, Baracchini *et al* 1988, McDowell *et al* 1988). The nucleotides of these bacterial cells were extracted (Little & Bremer 1982) and analysed by these methods. These methods of analysis of the bacterial extracts of nutrient limited cultures may be used to indicate the concentration of the HPNs present in the bacterial cells and the time of their production. The time at which these HPNs are produced may indicate if there is a direct link between their appearance in the cells and the simultaneous development of bacterial resistance to antibiotics.

Unfortunately, no commercial source of 'Magic Spot' was available to act as control in these experiments. The lack of 'Magic Spot' control meant that the published data of the  $R_f$  (2-D TLC; Randerath & Randerath 1964, Lagosky & Chang 1980, Bochner & Ames 1982a,b) and  $R_t$  values (HPLC; Ochi *et al* 1981, Little & Bremer 1982, Baracchini *et al* 1988, McDowell *et al* 1988) for 'Magic spot' were the only support for the experimental results obtained.

## Planar Chromatography

### *Thin Layer Chromatography (TLC)*

Planar chromatography is a form of liquid chromatography in which the stationary phase is supported upon a planar surface rather than in a column. There are two types of planar chromatography: paper chromatography (PC) and thin layer chromatography (TLC). PC preceded TLC by about 10-15 years, but TLC has superseded PC due to the improved development time, versatility and reproducibility of this technique. The separation of the mixture of compounds on plates produce sharper spot shapes and greater resolution than separation on paper (Fried & Sherma 1986, Robards *et al* 1994). In planar chromatography, the sample is applied as a spot or streak of minimal size to a marked position on the planar surface. After evaporation of the solvent, the plate is placed in a suitable sealed tank or chamber, with one end immersed in the solvent chosen as the mobile phase. The spot of sample is not immersed as it would dissolve off the plate into the solvent. The mobile phase percolates through the stationary phase by capillary action and moves the components of the sample in the direction of flow. This movement is dependent upon the chemical and ionic structure of the components to be separated and their affinity for both solvent and substrate. After the solvent has moved the required distance (solvent front), the chromatogram is removed from the chamber and dried. The separated components are located and characterised by the reference values ( $R_f$  value, Equation 5), (Berg 1963, Braithwaite & Smith 1985, Robards *et al* 1994).

(Equation 5)  $R_f = \text{distance moved by solute} / \text{distance moved by solvent front}$ .

### *Two Dimensional Thin Layer Chromatography (2D-TLC)*

Nucleic acid derivatives can be separated on a limited number of different sorbent products such as, cellulose, silica gel, polyamide, alumina and kieselgur. There are a large number of solvents and solvent mixtures available for TLC. The choice of mobile phase must be of a high purity and at a reasonable cost, non-reactive with the stationary phase and the components of the sample, and miscible with other solvent mixtures (Robards *et al* 1994). Nucleotides and their derivatives are detected using shortwave ultraviolet (UV) light (Fried & Sherma 1986). Maps of the spots are constructed following two-dimensional (2D) separation and visualisation under short wave UV light.

Significant studies of nucleic acid derivatives on cellulose were carried out by Randerath and Randerath (1964). They used poly(ethyleneimine)-cellulose thin layers (PEI-cellulose) to separate 23 ribonucleotides. Di- and trinucleotides were separated, as were some nucleotide sugars (Randerath & Randerath 1964, Fried & Sherma 1986).

Two dimensional thin layer chromatography (2D-TLC) is a simple method of increasing the effectiveness of chromatographic separations. The chromatogram is developed first in one direction. The plate is removed and dried, to evaporate the solvent. The plate is rotated through 90°, the lower edge is immersed in a second solvent and the chromatogram is further developed. The use of two different solvents and two different directions results in enhanced resolution efficiency. Solutes are easily detected if they are highly coloured or can absorb and re-emit UV radiation. The solutes being separated may either quench the natural fluorescence of the plate or fluoresce much more strongly than the plate. In either case the spots will stand out against the cellulose background. The introduction of sensitising sprays enhances the solutes thus, making identification easier and more accurate (Berg 1963). The methods of Randerath and Randerath (1964) resulted in a rapid analytical method for the separation and detection of nucleotides and their derivatives. This technique was particularly useful in the identification of components concerned with the composition and metabolism of nucleotides in samples extracted from bacterial, plant and animal cells.

#### *2D-TLC Analysis of Overnight Bacterial Cellular Nucleotides*

Aliquots (10 $\mu$ L) of cellular nucleotides obtained from overnight samples of *B. subtilis* and *E. coli* (page 68) were assayed for the presence of nucleotides using 2D-TLC (page 69). In order to enhance the cellular nucleotides present in the sample, the plates were sprayed with 10mM Tb(NO<sub>3</sub>)<sub>3</sub> which enhances the guanine nucleotides (Sy & Roselle 1981). The plates were then sprayed with 1.5M chloroacetaldehyde which enhanced the appearance of the adenine nucleotides present (Leonard *et al* 1972). The developed plates were examined under short wave UV light ( $\lambda$ 254nm) in order to determine the presence of highly phosphorylated nucleotides (Fig 21). The nucleotides eluted on the plates were traced and the *R<sub>f</sub>* values were calculated.

When *E. coli* extracts were developed in the first dimension there was no indication of individual separation of the nucleotides present in the sample. There was,

however, clear resolution of nuclear material under both visible and short wave UV light ( $\lambda 254\text{nm}$ ), apparent as a fluorescent streak developed from the point of origin. In the second dimension AMP, ADP and very small amounts of ATP and GTP were identified when their  $R_f$  values (Table 13) were compared to those obtained from the mixture of standard nucleotides (Table 7). A spot was observed directly below the ATP nucleotide standard which was probably pppApp.

**Table 13:** 2D-TLC  $R_f$  values of cellular extracts of *Escherichiacoli* NCIMB 10000 (10 $\mu$ L sample)

Dimension	$R_f$ value	Nucleotide
1 <sup>st</sup>	0.646	Mono, Di and Tri-nucleotides*
2 <sup>nd</sup>	0.6	AMP
	0.4433	GMP
	0.316	ADP
	0.24	GDP
	0.026	GTP
	0.0	ATP

Mono-nucleotides = AMP and GMP

Di-nucleotides = ADP and GDP

Tri-nucleotides = ATP and GTP

\* = Combined spot containing AMP, GMP, ADP, GDP, ATP and GTP

As the role of the HPN's ppApp and pppApp was thought to be different from that of ppGpp and pppGpp (Rhaese *et al* 1976), it was suggested that the pppApp may be involved in the control of amino acids as it's activity appeared to be exterior to the cell. ppApp and pppApp were isolated from the cellular extracts of *B. subtilis* alone. Therefore, it was

investigate only ppGpp and pppGpp in future experiments. There was a second spot directly below the standard nucleotide GDP was thought to be ppGpp (Bochner & Ames 1982a). The spot which corresponded to the AMP nucleotide standard was much larger than that for the other nucleotides present in the cellular extract. This is possibly the result of the donor ATP losing two phosphates during the production of MSI and MSII, resulting in an increase in AMP concentration (Fehr & Richter 1981(b), Beaman *et al* 1983, Cashel & Rudd 1987). It was not possible to positively identify the presence of “Magic Spot” using this method with *E. coli*. This may have been the result of the size and charge on the HPNs and the possibility that the ionic strength of the solvents were not strong enough to separate the HPNs from the cellular extract mixture.

The *B. subtilis* cellular extracts (Table 14; Fig 21) exhibited clear resolution when viewed under short wave UV light ( $\lambda$ 254nm). The development of the second

**Table 14:** 2D-TLC *R<sub>f</sub>* values of cellular extracts of *Bacillus subtilis* NCIMB 10000 (10 $\mu$ L sample)

Dimension	<i>R<sub>f</sub></i> value	Nucleotide
1 <sup>st</sup>	0.585	Mono, Di and Tri-nucleotides*
2 <sup>nd</sup>	0.9189	Mononucleotides
	0.466	ADP
	0.135	GDP
	0.013	MSI
	0.01	MSII

Mono-nucleotides = Combined spot containing both AMP and GMP

Di-nucleotides = ADP and GDP

Tri-nucleotides = ATP and GTP

\* = Combined spot containing AMP, GMP, ADP, GDP, ATP and GTP

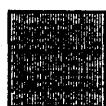
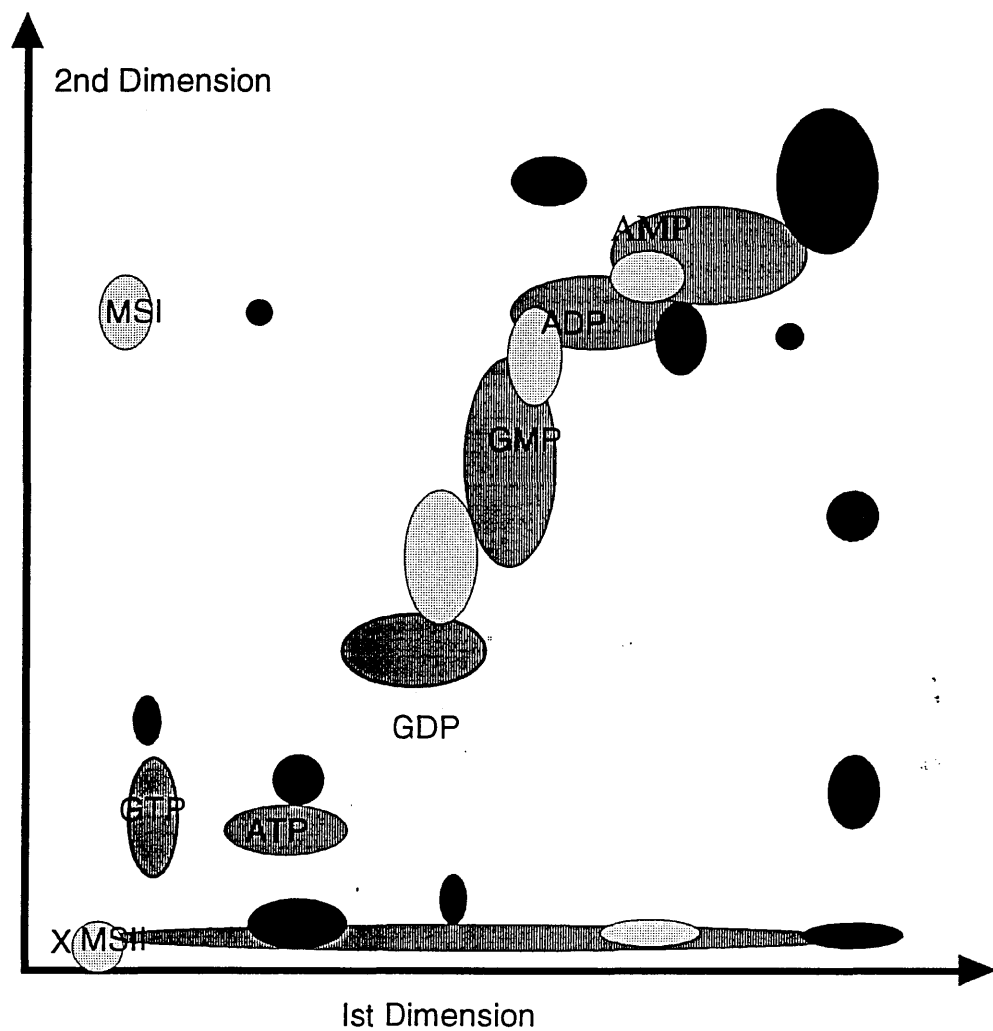


dimension separated the nucleotides. These nucleotides were difficult to identify as the observed  $R_f$  values were slightly different from the standard  $R_f$  values (Table 7).

The monophosphates (AMP, GMP) were present, but it was impossible to positively identify them. ADP and GDP were identified in small quantities, but there was no detectable ATP or GTP. The *B. subtilis* cellular extracts exhibited an area of fluorescence at the point of origin after the second dimension development of the plate. This spot was not visible after the first dimension development. It was suggested that this area of fluorescence indicated the presence of “Magic Spot”. During the production of ‘Magic Spot’ the concentration of ATP may be reduced or the nucleotide may disappear entirely, due to the pyrophosphate transfer to GTP. Thus, converting GTP to pppGpp. This may result in the reduction in concentration or disappearance of the nucleotide GTP. The inability to detect the HPN’s after the first dimension development may be due to the compounds being too large to be separated from the cellular extract mixture. The lack of detection may also be due to the solvent used for first development only being suitable for the separation of mono-, di- and tri-nucleotides and not the enzymatically synthesised HPN’s. The reduction of the content of both ADP and GDP along with the disappearance of both ATP and GTP is indicative of the ability of *B. subtilis* to produce both types of HPN and thus, ATP can be the acceptor as well as the donor nucleotide and ADP, GDP and GTP are nucleotide acceptors.

2D-TLC was a useful method in the qualitative identification of some of the major nucleotides present in the bacterial extract samples, but it proved difficult to positively identify all the particular nucleotides of interest. It was necessary therefore, to employ a rapid technique which would identify the presence and concentrations of HPN’s in the bacterial extracts. Further literature review suggested that HPLC would be a suitable method of separation. Separation of HPN’s from bacterial extracts had been performed using ion-pair HPLC. Ochi *et al* (1981) and Payne and Ames (1982) separated ‘Magic Spot’ from bacterial extracts using a Partisil PXS 10/25 SAX [Whatman],  $\text{SO}_4^{2-}$  form column. Little and Bremer (1982) and Baracchini (1988) successfully separated ‘Magic Spot’ using ion-pair reverse-phase HPLC, in which the column employed was an Ultrasphere-IP column (4.6mm x 25cm) with a  $\text{C}_{18}$  precolumn.

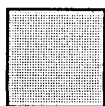
**Figure 21:** 2D-TLC of 20 $\mu$ L controls and 20 $\mu$ L cell extracts of *Bacillus subtilis* NCIMB12900 and *Escherichia coli* NCIMB 10000 (Randerath & Randerath 1964).



Nucleotide standards  
(20 mg/ml in 1M LiCl)



*Escherichia coli* NCMB  
10000 cell extract



*Bacillus subtilis* NCIMB  
12900 cell extract

### *High Performance Liquid Chromatography (HPLC).*

High performance liquid chromatography (HPLC) is an ideal technique for the analysis of small molecules with a molecular weight less than 1000 Daltons. There are various modes of separation available in this technique, including gel-permeation (GPC), adsorption (normal phase or liquid-solid), partition (liquid-liquid), ion-exchange and reversed-phase (RP) chromatography (Lim 1986). Ion-exchange chromatography columns are usually composed of silica, chemically bonded with anionic or cationic groups (commonly aminopropyl, tetra-alkylammonium or sulphonic groups; Wood *et al* 1980, Braithwaite & Smith 1985).

The separation of nucleotides, nucleosides and bases has occupied the central role in the development of HPLC. There are a wide variety of approaches to the analysis of nucleic acid components. Many of these approaches rely upon the characteristic UV spectra of the compounds. The complexity and diversity of nucleotides found in living organisms determines the necessity for a chromatographic approach for their analysis (Perrett 1986). Ion-exchange chromatography separates molecules on the basis of their molecular charge. The separation proceeds because ions of opposite charge are retained by the column to different extents. The resolution of separation is influenced by the pH of the eluent, which affects the selectivity, and by the ionic strength of the buffer, which mainly affects the retention time (Lim 1986). Due to their phosphate ester linkages, nucleotides are highly charged when in the pH range 2-7, the useful pH range for silica-based stationary phases. Over the pH range 1-5, the charge directly increases with the number of phosphate residues; ie. AMP < ADP < ATP. The gross level of charge is modified by the subsequent phosphate groups on the heterocyclic ring, this and the pH determine the elution order of the compounds from the anion-exchange column. Ion-exchange chromatography has provided a useful approach to the resolution of nucleotides in complex mixtures. Elution from anion-exchange resins using increasing salt gradients has been employed since the very earliest days of column chromatography (Perrett 1986). Nucleotides can be separated on all available silica-based microparticulate anion-exchangers. These can be the weak ammonium variety; eg. aminopropylsilane (Si-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-NH<sub>2</sub>, APS) or the strong quaternary variety (SAX), eg. trimethylaminopropyl [Si-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-N<sup>+</sup>(CH<sub>3</sub>) Cl<sup>-</sup>]. The amine groups in a weak ion-exchanger, are fully charged over the pH range 1 to 7, whereas the strong ion-exchanger has an extended usable range from a pH of 1 to 12 (Perrett 1986, Robards *et al*

1994). Ion-exchange chromatography is an important analytical method for the separation of organic and inorganic solutes and is important for the separation of amino acids and proteins. To date HPLC is the chosen approach for both qualitative screening and quantitative analysis of nucleotides from complex mixtures.

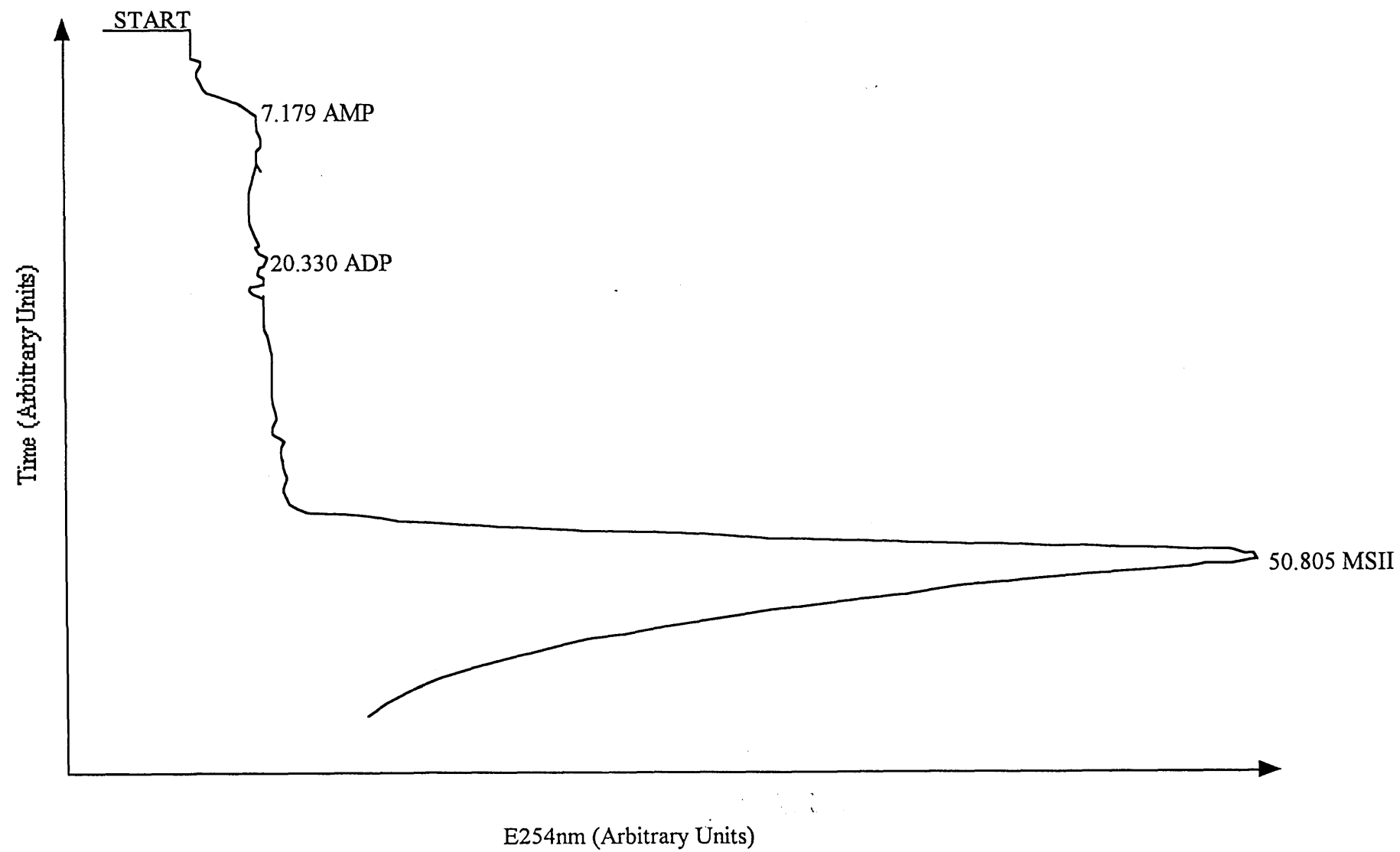
#### *Preparation of Standard Nucleotides*

Aliquots (20 $\mu$ L) of nucleotides (either AMP, GMP, ADP, GDP, ATP or GTP at concentrations of  $1 \times 10^{-12}$  mg mL<sup>-1</sup> in sterile dH<sub>2</sub>O) were assayed using method of Ochi *et al* (1981) and Little and Bremer (1982). The nucleotides were eluted using a gradient buffer system over 65 minutes (Table 8) at a flow rate of 1.5 mL min<sup>-1</sup>.

The gradient HPLC system used a low ionic strength buffer, 7mM KH<sub>2</sub>PO<sub>4</sub> (pH4 adjusted with 1M H<sub>3</sub>PO<sub>4</sub>) and a high ionic strength buffer, 0.5M KH<sub>2</sub>PO<sub>4</sub> + 0.5M Na<sub>2</sub>SO<sub>4</sub> (pH5 adjusted with 1M KOH). The buffers were made up in sterile dH<sub>2</sub>O, filtered using 0.22 $\mu$ m (Whatmans) nitrocellulose filter paper, and degassed for 30 minutes using a vacuum pump. Aliquots (20 $\mu$ L) of the nucleotide standards were loaded onto a Partisil PXS 10/25 SAX (Whatman), SO<sub>4</sub><sup>2-</sup> form column and were eluted using a Spectraphysics SP8800 Ternary HPLC Pump, Hewlett & Packard HP3396 Series 911 Integrator and Hewlett & Packard Series 1050 UV Detector. The retention time ( $R_t$ ) of each individual nucleotide was determined. The  $R_t$  (Table 9) for a mixture of all 6 nucleotides in an equimolar concentration was also determined by the same techniques (Fig 10).

HPLC is capable of detecting low concentrations of compounds (Figures 22 & 23) and was the obvious choice for the assays of the bacterial cellular extracts. Initial experimental work was performed with standard nucleotides, with sample concentrations ranging from 1mg mL<sup>-1</sup> to 10mg mL<sup>-1</sup>, in order to obtain their  $R_t$  values (Table 9; Fig.10). Once the control  $R_t$  values were obtained the experimental work proceeded with cellular samples extracted from overnight cultures of *E. coli* and *B. subtilis*. The cellular extracts were assayed using ion exchange HPLC. The  $R_t$  values were observed and noted. In both bacteria, the traces displayed a reduction in the levels of ATP, GTP, ADP and GDP. There were peaks which corresponded to MSI and MSII (Table 15; Figs. 22 & 23, Ochi *et al* 1982). These peaks were much larger than the peaks obtained using standard nucleotides,

**Figure 22:** HPLC trace of a 20 $\mu$ L aliquot of cellular extract from an overnight culture of *B. subtilis* NCIMB 12900 eluted over a 65 minute gradient (Ochi *et al* 1981), flow rate 1.5mL min<sup>-1</sup>, with 7mM KH<sub>2</sub>PO<sub>4</sub> (pH4) and 0.5M Na<sub>2</sub> SO<sub>4</sub> + 0.5M KH<sub>2</sub>PO<sub>4</sub> (pH5).



**Figure 23:** HPLC trace of a 20 $\mu$ L aliquot of cellular extract from an overnight culture of *E. coli* NCIMB 10000 eluted over a 65 minute gradient (Ochi *et al* 1981), flow rate 1.5mL min<sup>-1</sup>, with 7mM KH<sub>2</sub>PO<sub>4</sub> (pH4) and 0.5M Na<sub>2</sub> SO<sub>4</sub> + 0.5M KH<sub>2</sub>PO<sub>4</sub> (pH5).





A large peak produced at approximately 52 minutes during the analysis of *E. coli* was thought to be 'Magic Spot', as it corresponded to the published data of Ochi *et al* (1981). This peak appeared on the trace of every sample analysed by this method. This peak was thought to have been an excess of HPN which had bound to the stationary-phase within the column and could not be completely eluted. Alternatively, it may have been a protein residue from the bacterial extract. In order to determine if the peak was a protein contaminant or a bound HPN, the fraction corresponding to this peak was collected and analysed. The protein content of the fraction was measured using the Lowry test (Lowry *et al* 1951; Fig 11) and the nucleotide content of the fraction was measured according to the protocol of Adams *et al* (1992). The Lowry test indicated that there was no protein present in the sample thus, eliminating protein as the problem.

**Table 15:** Retention times of standard nucleotides and bacterial cellular extracts of *Escherichia coli* NCIMB10000 and *Bacillus subtilis* NCIMB 12900

Nucleotides [10mg mL <sup>-1</sup> ] 20μL	Standard Retention time R <sub>t</sub> (mins)	<i>E. coli</i> R <sub>t</sub> (mins)	<i>B. subtilis</i> R <sub>t</sub> (mins)
AMP	9.105	8.555	7.179
GMP	11.958	11.640	/
ADP	21.228	19.521	20.330
GDP	30.141	30.955	/
ATP	49.700	43.444	/
GTP	51.389	/	/
ppGpp	52.00*	52.98	50.805
pppGpp	60.00*	55.030	/

/ = not found

\* Published data (Ochi *et al* 1981)

In order to determine the nucleotide concentration of the fraction an estimation by extinction at E260nm and E280nm (Adams *et al* 1992) was performed. The optical densities at these wavelengths were observed and noted. The absorbancy of the sample at E260nm was 0.019 and 0.014 at E280nm. The ratio between the two optical densities is indicative of the nucleotide concentration (Adams *et al* 1992; Equation 6). Using the table provided (Adam *et al* 1992) for estimation of the presence of nucleotide, 0.7368 indicated that the nucleotide content was between 8.5 and 9.3%. These findings suggest that there was no protein present in the fraction, but there were nucleotides.

(Equation 6)

$$\begin{aligned} n &= E280nm / E260nm \\ &= 0.014 / 0.019 \\ &= \underline{0.7368} \end{aligned}$$

This indicated that the peak produced at 52 minutes during the analysis of *E. coli* contained ‘Magic Spot’ and that it had formed a strong bond to the column. The buffers utilised to elute the nucleotides from the column appeared to be unable to remove the HPN from the column completely. In order to remove the residual ‘Magic Spot’ from the column, a specialist cleaning and regenerating regime was undertaken after every sample. The specialist cleaning regime was provided by Whatmans, the manufacturer of the column, and consisted of pumping 7mM KH<sub>2</sub>PO<sub>4</sub> through the column for one hour. This was followed by a one hour wash with sterile dH<sub>2</sub>O. The column was then acid washed for one hour with 0.01M H<sub>2</sub>SO<sub>4</sub>, and further rinsed with sterile dH<sub>2</sub>O for one hour. In order to remove the loosened components of the bacterial sample the column was washed with a chelating agent 0.1M EDTA for one hour and then rinsed again for one hour with sterile dH<sub>2</sub>O. The removal of adsorbed organics from the bonded organic phase was achieved by a methanol wash for one hour. The column was then rinsed for one hour with sterile dH<sub>2</sub>O, in order to prevent the precipitation of the phosphates present in the HPLC eluents. The column was then equilibrated with low ionic strength buffer (7mM KH<sub>2</sub>PO<sub>4</sub>). After this special cleaning regime was performed, the peak at 52 minutes was still present in the traces obtained from the HPLC system. In order to solve this problematic peak at 52 minutes one parameter utilised within the system was changed. The easiest change was to try alternative eluents and with the same column.

Payne and Ames (1982) separated HPNs from bacterial cellular extracts using the same type of column (Partisil PXS 10/25 SAX (Whatman),  $\text{SO}_4^{2-}$  form), with alternative eluents and gradient programmes. The low ionic strength eluent, 40mM ammonium acetate (pH adjusted to 6.0 with 0.1M HCl) and the second high ionic strength eluent, 3.0M ammonium acetate (pH adjusted to 6.0 with 0.1M HCl) were prepared with sterile  $\text{dH}_2\text{O}$ , filtered using  $0.22\mu\text{m}$  (Whatmans) nitrocellulose filter paper, and degassed for 30 minutes using a vacuum pump. Payne and Ames (1982) suggested a 45 minute gradient curve No 7 with a flow rate of  $1\text{mL min}^{-1}$ . The published paper of Payne and Ames (1982) failed to disclose the details concerning gradient No 7, therefore, the gradient time used previously was substituted (Ochi *et al* 1981). The observed retention times of the nucleotides did not correspond to the times of the results published by Payne and Ames (1982). As the gradient programme of Ochi *et al* (1981) required extra time required (65 minutes as opposed to 45 minutes according to Payne and Ames (1982)). The extra time was included in the calculation of the retention times. The retention times of the nucleotides obtained by Payne and Ames (1982) were not comparable with the results of Ochi *et al* (1981). Therefore, various gradient times were utilised from information obtained in the literature concerning HPLC methods (Ochi *et al* 1981, Little & Bremer 1982, Baracchini *et al* 1988, McDowell *et al* 1988). The gradient times utilised from the information obtained from the literature survey did not produce comparable  $R_t$  values for nucleotides when separated with the eluents of Payne and Ames (1982).

Due to the strongly bound HPNs to the Partisil PXS 10/25 SAX (Whatman),  $\text{SO}_4^{2-}$  form column. The separation of the nucleotides was performed according to the protocol of (Little & Bremer 1982, Baracchini 1988, Supelco Chromatography Products 1996) utilising reverse-phase chromatography (RF). The stationary-phase in reverse-phase chromatography is silica chemically bonded with an alkylsil compound to give a non-polar, hydrophobic surface. The retention of the solute tends to be mainly due to hydrophobic interactions between the solutes and the hydrocarbonous stationary phase surface. Polar mobile phases used for elution, tend to be water mixed with methanol, acetonitrile and/or other water-miscible organic solvents. Solutes are eluted in order of decreasing polarity (increasing hydrophobicity), and increasing the polar (aqueous) component of the mobile phase increases the retention of the solute (Lim 1986, Robards *et al* 1994). The most popular

type of packing in column used in RF column is C<sub>18</sub> type in which octadecylsilyl (ODS) groups are bonded to the silica surface. Silica with C<sub>22</sub>, C<sub>8</sub>, C<sub>4</sub>, C<sub>3</sub>, C<sub>2</sub> and C<sub>1</sub> groups attached are also available. The retention times of solutes usually increase proportionally with the length of the carbon chain of the bonded groups under identical HPLC conditions (Lim 1986, Robards *et al* 1994). The column required for this method was a Supelcosil LC-18-T (15cm x 4.6mm 3µm particle size). The buffers were prepared with sterile dH<sub>2</sub>O, filtered using 0.22µm (Whatmans) cellulose acetate filter paper, and degassed for 30 minutes using a vacuum pump. The elution buffers prepared were 0.1M potassium dihydrogen phosphate (pH adjusted to 6.0 with 1M H<sub>3</sub>PO<sub>4</sub>) to this 8mM tetrabutylammonium sulfate was added (buffer A). The second buffer was a mixture of buffer A and methanol, at a ratio of 70%:30% respectively (pH adjusted to 5.5 with 1M H<sub>3</sub>PO<sub>4</sub>; buffer B). The flow rate employed for the elution of the nucleotides was 1.5mL min<sup>-1</sup>. The retention times of the nucleotides were very similar to each other, there was no clear separation and this made their identification particularly difficult (Table 16).

The original ion-exchange system was repeated, as it originally produced clear precise peaks, before the HPN's became irreversibly bound to the stationary phase of the column. The Partisil PXS 10/25 SAX (Whatman), SO<sub>4</sub><sup>2-</sup> form column ion-exchange system was set up and recalibrated.

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**Table 16:** Gradient programme for reverse-phase HPLC

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Time (mins)	% Buffer A	% Buffer B
0 - 2.5	100	0
5.0	80	20
10	60	40
13 - 18	0	100
18.1 - 22	100	0

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The large peak at 52 minutes re-appeared during the calibration of the column with the standard nucleotides, indicating that it was not due to the presence of 'Magic Spot'. The appearance of this large peak may have been due to the shift in base line due to the change over from low ionic strength buffer to high ionic strength buffer. This was highly problematic as the shift in the base line occurred at approximately the same time as the expected elution of the HPN's would inevitably mask the peaks. A research group working in a similar area were contacted concerning this problem and it was revealed that they also experienced the same difficulties (England, R. University of Central Lancashire, Personal Communication). They also confirmed the theory that the peak at 52 minutes was probably due to the shift in the base line as a result of the switching over from low ionic strength buffer to high ionic strength buffer.

It was determined that the HPN's of interest were separated with high ionic strength buffer during the methods of Ochi *et al* (1981). Therefore a new method was devised employing an isocratic system and using high ionic strength buffer as the mobile phase. Jones *et al* (1996) had used a standard control of 'Magic Spot' and were contacted to find out where they obtained it (England, R. University of Central Lancashire, Personal Communication), and if they could possibly donate some. They were provided with a sample by the Mercian Corporation, Japan, but were not in a position to supply the standard to us as they had a very small supply. The Mercian Corporation were contacted, but they were also unable to supply the HPN's.

The unavailability of a commercial standard of ppGpp or pppGpp made it necessary to enzymically produce the standard in the laboratory. Murao and Nishino (1973) had isolated the enzyme, Nucleotide Pyrophosphotransferase, from a bacterium isolated in soil, which produced 'Magic Spot' (Murao & Nishino 1973). The bacterium *Streptomyces adepospholyticus* A 4668 was obtained from the Institute for Fermentation (IFO), Osaka, Japan via National Collections of Industrial and Marine Bacteria (NCIMB), Aberdeen, UK. The bacterium was catalogued as *Streptomyces adepospholyticus* A-4668 NCIMB 13429. The production of a control of 'Magic Spot' was performed in the laboratory according to the protocol of Murao and Nishino (1974).

## Nucleotide Pyrophosphotransferase Enzyme Production

Nucleoside phosphotransferase, which synthesises nucleotides by the transfer of pyrophosphate from organic nucleoside to GDP and GTP, was reported by Brawerman and Chargaff (1952). Mitsugi *et al* (1964) suggested that nucleoside phosphotransferase was widely distributed in bacteria as an intracellular enzyme. The transfer of the terminal phosphoryl groups to a nucleotide acceptor has been well investigated (Cashel & Gallant 1969, Block & Haseltine 1974, Chaloner-Larsson & Yamazaki 1975, Gallant & Lazzarini 1976, Cashel & Rudd 1987, O'Farrell 1978, Lagosky & Chang 1980, Fehr & Richter 1981(a), Kelly *et al* 1991, Gustafson *et al* 1993, Kjelleberg *et al* 1993), but there has been little research into the occurrence of  $\beta$ - $\gamma$ -pyrophosphate transfer of nucleoside triphosphate to a nucleoside or nucleotide. Haseltine *et al* (1972) and Pederson *et al* (1973) reported that the stringent factor contained in the ribosomal wash of *Escherichia coli* stimulated the synthesis of the HPNs ppGpp and pppGpp, through enzymic pyrophosphoryl transfer from ATP to GDP and GTP. This reaction, catalysed by the stringent factor, appears to be dependent upon ribosomes. The enzyme ATP: nucleotide pyrophosphotransferase catalyses the synthesis of new nucleotides, such as pppApp, ppApp, pppGpp or ppGpp from nucleoside-5'-mono (di- and tri-) phosphate as pyrophosphate acceptor and ATP as pyrophosphate donor (Murao & Nishino 1974). It appears that only purine nucleotides are involved in the enzymatic production of 'Magic Spot'. The production of 'Magic Spot' was performed using the culture filtrate of *Streptomyces adenosiphilicus* and the nucleotide ATP. The enzyme requires  $Mg^{2+}$  ions for its activity and is most effective at a pH range of 9.5 to 10.0. Nishino and Murao (1975) suggested that the substrate for the enzyme is a metal-ATP complex. The enzymic activity obtained using  $Mg^{2+}$  as a cofactor was higher than that observed with other metal ions utilised (Nishino & Murao 1975).

### *Nucleotide Pyrophosphotransferase Production.*

The nucleotide pyrophosphotransferase was produced according to the method of Murao and Nishino (1974). The medium (page 61) was inoculated and incubated (page 61). The resultant culture was centrifuged (10,000xg, 10 min) and the supernatant was filtered through 0.22 $\mu$ m (Whatman) sterile cellulose acetate filter to remove cell debris. The supernatant was stored on ice until required.

**Determination of Enzymic Activity by the Estimation of Phosphate Concentration**

*Construction of Phosphate Calibration Curve*

It was necessary to determine the concentration of both total and labile phosphate in standards solutions, with known phosphate concentrations. These phosphate standards were analysed by the method of Fiske and Subbarow (1957). A calibration curve was constructed using the results obtained from the experiments utilising the phosphate standard solutions (Fig 24). Both total and labile phosphate concentrations of the culture filtrate could then be estimated from this calibration curve.

*Phosphate Determination of Culture Filtrate.*

The concentrations of labile phosphate and total phosphate in the culture filtrate were determined using the method of Fiske and Subbarow (1957) replacing the phosphate standard solutions with aliquots (1mL) of the culture filtrate. The phosphate concentrations of the culture filtrate were determined from the phosphate standard calibration curve (Fig 24, Table 17). The level of labile phosphate present in the sample was used to estimate the amount of AMP produced as a by-product of the nucleotide pyrophosphotransfer.

**Table 17:** Phosphate content (labile and total) of culture filtrate of *S. adepospholyticus*.

Phosphate	E660nm	Phosphate concentration (from calibration curve)
Labile	0.646	$1.5 \times 10^{-4} \text{M}$
Total	2.175	$5.857 \times 10^{-3} \text{M}$



### Assay of Enzyme Activity

Enzyme activity was estimated by the determination of the concentration of labile and extralabile phosphate (pppApp) (Nishino & Murao 1975). The concentration of AMP present in the culture filtrate determined from the calibration curve, was used to estimate the activity of the nucleotide pyrophosphotransferase (Fiske & Subbarow 1957, Hamagishi *et al* 1975). The enzyme in the culture filtrate was incubated for 10 minutes with a reaction mixture containing glycine-NaOH buffer (pH10), ATP, GTP and  $Mg^{2+}$  (Table 18). The enzyme activity was determined by comparison of the concentration of total phosphate and labile phosphate. The comparison of these samples was observed at E660nm using a Cecil CE Series 2 Spectrophotometer blanked against a 2mL sample of sterile dH<sub>2</sub>O in a 1cm path length cuvette. The determination of the enzyme activity was used to calculate the volume of the culture filtrate required to provide 6 units of enzyme (1 unit of enzyme activity was defined as the amount of enzyme forming 1 $\mu$ Mole AMP min<sup>-1</sup> at 37°C) (Nishino & Murao 1975).

**Table 18:** Composition of enzymatic reaction mixture  
(Hamagishi *et al* 1975).

Component	Concentration
glycine buffer	1.5mmole (pH10)
ATP (donor nucleotide)	200 $\mu$ mole
GTP (acceptor nucleotide)	200 $\mu$ mole
MgCl <sub>2</sub>	200 $\mu$ mole
enzyme	6 units
pH	10.0

### *Enzymic Synthesis of ppGpp and pppGpp Standards.*

The production of ppGpp and pppGpp standards was performed according to the protocol of Hamagishi *et al* (1975) using the nucleotide pyrophosphotransferase enzyme. Aliquots (24mL) of reaction mixture were prepared in sterile deionised Erlenmyer flasks (100mL) (Table 18). These were incubated for 4 hours at 30°C, 200 osc min<sup>-1</sup>. The pH of the reaction mixture was adjusted to pH 7.0 with 1.0M HCl. This was then filtered using 0.22µm (Whatmans) sterile cellulose acetate filter paper. The sample was then analysed for the presence of the HPNs using FPLC (Horrocks, AJH. University of Abertay Dundee, Personal Communication).

### **Fast Protein Liquid Chromatography (FPLC)**

#### *Buffer preparation*

Tris-HCl; (0.05M; pH 7.5; Buffer A) and LiCl; (1M in 0.05M Tris-HCl; pH 7.5; Buffer B) were prepared in sterilised dH<sub>2</sub>O. The buffers were then filtered using 0.22µm (Whatmans) cellulose acetate filter paper, and degassed for 30 minutes using a vacuum pump.

#### *Analysis of Enzyme Reaction Mixture*

The presence of ppGpp and pppGpp was assayed using FPLC (Horrocks, A.J.H. University of Abertay Dundee, Personal Communication). Aliquots of the reaction mixture (500µL) were injected onto a Pharmacia FPLC mono S column. The nucleotides were eluted using a salt gradient (Table 19) ranging from 0M to 1M LiCl in Tris-HCL, over 75 mins at a flow rate of 1mL min<sup>-1</sup>. The Pharmacia FPLC system, Pump P-500, Gradient Programmer GP-250, Single Path Monitor UV-1 optical unit, mixer 5MPa, chart recorder were used to observe and record the results (Fig 25). Fractions of 1mL were collected using a Fraction Collector FRAC-100.

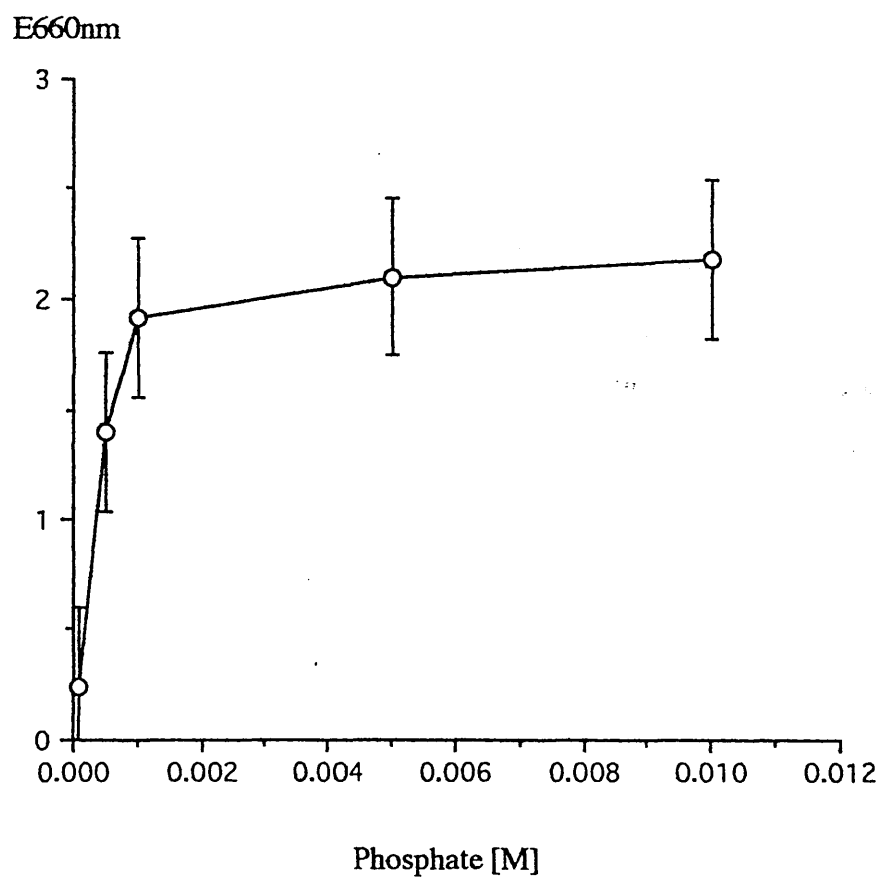
Hamagishi *et al* (1975) isolated ppGpp and pppGpp using a DEAE-Sephadex A-25 column. The HPNs were eluted at a salt concentration of 0.22 to 0.25M LiCl. The FPLC system eluted the nucleotides at LiCl concentrations which approximated 0.27M. The peaks produced using FPLC corresponded to the published data of Hamagishi *et al* (1975) thus, suggesting that the peak produced in fraction number 41 and eluted at a LiCl concentration of

0.27M, contained ‘Magic Spot II’ and fraction 35 contained ‘Magic Spot I’ (Fig 25). The fractions obtained during FPLC were stored at -20°C until required for HPLC analysis.

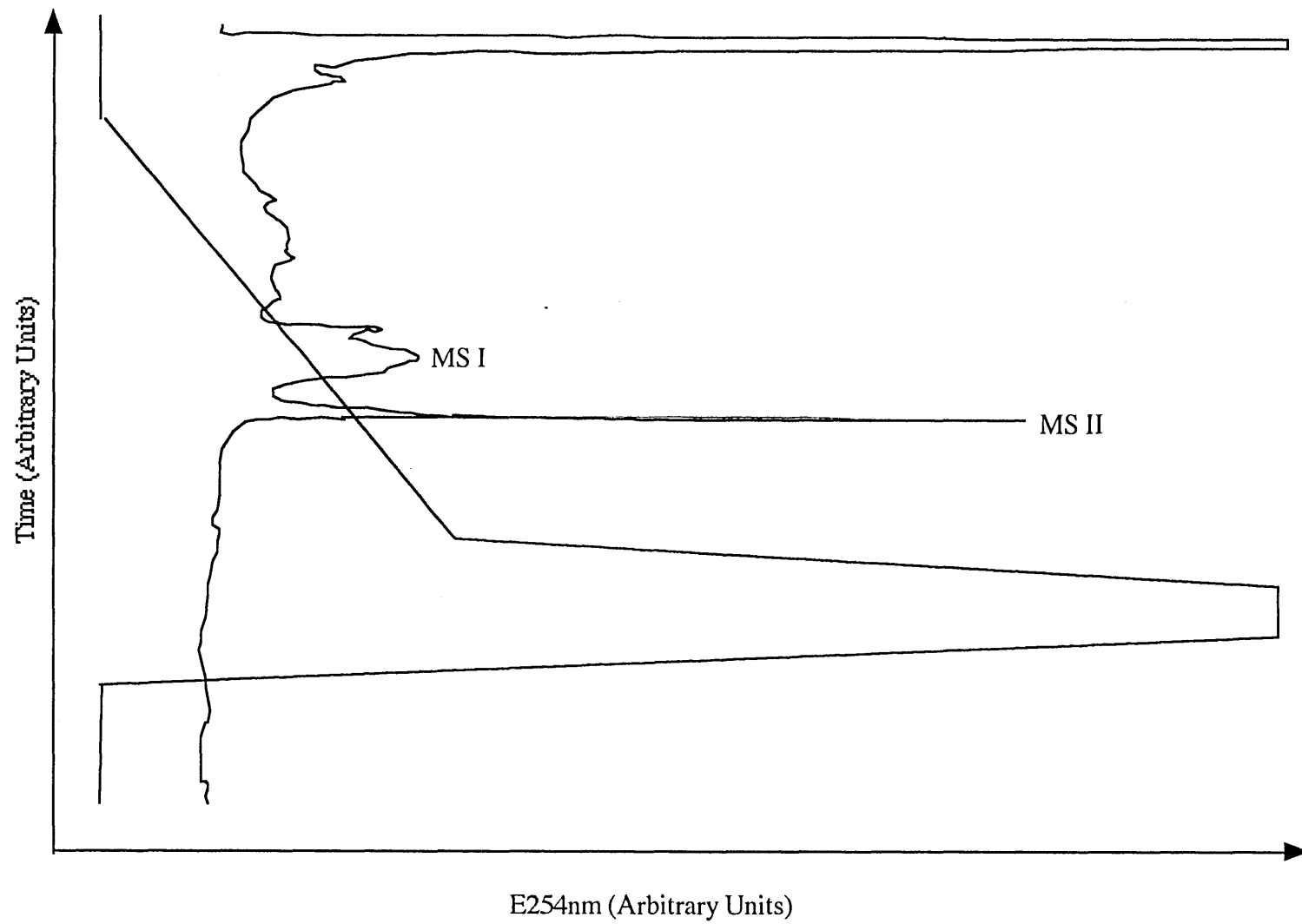
**Table 19:** FPLC Buffer Gradient Time

Time (mins)	%Buffer A	% Buffer B
10	90	10
50	70	30
54	0	100
59	0	100
64	100	0
74	100	0

**Figure 24:** Phosphate standard calibration curve concentrations (o) facilitating the determination of the concentrations of labile phosphate and total phosphate in culture filtrate of an enzyme preparation of *S. adepospholyticus* NCIMB 13429.



**Figure 25:** FPLC trace of MS I and MS II produced by pyrophosphotransferase enzyme in *S. adrophospholyticus* culture filtrate reaction mixture.



## Conclusion

The techniques employed in this project to identify 'Magic Spot' encountered many problems. The main problem was the lack of a suitable, commercially available 'Magic Spot' control. This was resolved, by the laboratory synthesis of the enzyme responsible for the production of 'Magic Spot' and its subsequent utilisation for the production of 'Magic Spot' (Hamagishi *et al* 1975).

2D-TLC was a useful method in the identification of the nucleotides present in the bacterial cellular extracts. A certain degree of separation was achieved and the technique exhibited that the nucleotides of interest either disappeared or that the concentration of the nucleotides was significantly reduced. A fluorescent spot at the origin of *B. subtilis* indicated the presence of 'Magic Spot' (Fig 21). The quantification of the nucleotides present was however, difficult as this required either radio labelling with the isotope  $P^{32}$ , or the isolation of the spot and its subsequent quantification by some other technique. Therefore, a rapid technique which would be both quantitative and qualitative was far more desirable. The use of HPLC appeared from the literature survey, to be an ideal method for the analysis of the nucleotides of interest. However, this method also proved to be problematic. The main re-occurring problem experienced when using the ion-exchange system was the shifting of the base line. The shift in the base line was due to the gradient programme switching buffers from low ionic strength to high ionic strength, in order to elute the HPNs. The time at which the base line shifted coincided with the published retention time (Ochi *et al* 1981), at which 'Magic Spot' in the bacterial extract samples would appear and therefore, lead to a masking in its appearance.

Alternative systems were tried, including different buffers with the same ion-exchange column, but the  $R_t$  values with the new buffers were not consistent and repeatability of results was not observed. Therefore, it was decided to utilise an entirely new HPLC system. The new system was a reverse-phase HPLC column with the appropriate buffers, but the retention times of the nucleotides eluted by this system were very similar to one another thus, positive identification of these nucleotides was unreliable.

The original system utilised a gradient programme in order to elute the nucleotides. The problems encountered with this system occurred when only the high ionic strength buffer was being pumped through the column, thus causing the shift in the base line. The high ionic strength buffer was the only buffer utilised as the mobile phase after 45



minutes of the gradient programme. According to the finding of Ochi *et al* (1981) the nucleotides of interest were expected to be eluted at 52 minutes and 55 minutes. As the high ionic strength buffer was the buffer which was employed at these times, an isocratic system was devised utilising the high ionic strength buffer. It was anticipated that the base line would not shift as there was only one buffer being pumped through the column. The retention times of the standard nucleotides and the enzymatically produced HPNs would have to be re-evaluated and the column re-calibrated with the system. The isocratic system appeared to have resolved the problem, in that there was no shift in the base line and the standard nucleotides were eluted with repeatable  $R_t$  values which were individually identifiable. The enzymatically produced 'Magic Spot' was eluted at a different  $R_t$  value than the other nucleotides thus, could be utilised as a 'Magic Spot' control.

# **THE GROWTH INHIBITORY AND ANTIMICROBIAL ACTIVITY of ANTIBIOTICS AGAINST *Escherichia coli* NCIMB 1000 and *Bacillus subtilis* NCIMB 12900**

## **CHOICE OF ANTIBIOTICS**

Four antibiotics were chosen to for use in this series of experiments, they were ampicillin (a  $\beta$ -lactam), streptomycin (an aminoglycoside), nalidixic acid (a 4-quinolone) and erythromycin (a macrolide). Streptomycin, ampicillin and nalidixic acid are effective against both Gram-positive and Gram-negative bacteria and erythromycin is most effective against Gram-positive bacteria, but somewhat less so against Gram-negative bacteria (Russell & Chopra 1990). Streptomycin and erythromycin target various sites on the ribosome, ultimately inhibiting protein synthesis. Nalidixic acid inhibits DNA gyrase and thus, inhibiting DNA replication. Ampicillin inhibits the final stage of peptidoglycan synthesis, transpeptidation.  $\beta$ -lactams bind to penicillin binding proteins (PBP's). These PBP's catalyse transpeptidase, carboxypeptidase and endopeptidase reactions. The primary target of  $\beta$ -lactams is the inhibition of the transpeptidation reaction which cross-links neighbouring glycan strands, which prevents further peptidoglycan synthesis thus, leading to cell death by lysis in hypoosmotic solutions.

## **Protein Synthesis**

Protein biosynthesis involves the use of mRNA in three stages; initiation, elongation and termination.

### *Initiation*

The N-terminal amino acid for all proteins is formylmethionine (fMet). fMet and the appropriate tRNA are united under the direction of an aminoacyl tRNA-synthetase thus, forming aminoacyl-tRNA. mRNA becomes attached to the 30S subunit of the ribosome, this attachment requires a soluble protein, F3 (initiation factor). The fMet- charged tRNA then combines with the mRNA-30S ribosomal complex. The avicodon triplet portion of the tRNA

is placed next, or close to the initiation codon in the mRNA [(5') AUG (3')]. Two additional initiation factors (F1 & F2) are required, as is GTP. GTP hydrolysis is required for the binding of amino-acyl-tRNA, the movement of peptidyl-tRNA from the aminoacyl (A) site to the peptidyl (P) site and the associated movement of the mRNA to the next codon. The 50S ribosomal subunit then binds to the mRNA-30S-tRNA-amino acid complex, the bound GTP is hydrolysed, and the initiation factors are released (Stryer 1975).

### *Chain elongation*

Elongation begins when a charged tRNA binds to the A site on the ribosome. A peptide bond is formed between the amino group of the incoming aminoacyl-tRNA and the carboxyl group of the the  $\mu$ Met carried by the initiator tRNA. This results in the formation of dipeptidyl-tRNA which is then translocated from the A site to the P site, whereas the other tRNA leaves the ribosome (Stryer 1975).

### *Termination*

Termination occurs when a stop codon is read by a protein release factor resulting in the release of the completed polypeptide chain from the ribosome. Aminoacyl-tRNA does not normally bind to the A site of a ribosome if the codon is UAA, UGA or UAG. Normal cells do not contain tRNAs with complementary anticodons to the stop signals. These stop signals are recognised by release factors, which are proteins. The binding of a release factor to a termination codon in the A site activates peptidyl transferase which results in the hydrolysis of the bond between the polypeptide and the tRNA in the P site. Thus, the polypeptide chain leaves the ribosome. The 70S ribosome then dissociates into 30S and 50S subunits as the prelude to the synthesis of another protein (Stryer 1975).

### *Effect of Antibiotics upon Protein Synthesis*

Aminoglycosides are most effective against Gram-negative bacteria. Their main target is the ribosome. Aminoglycoside-ribosome interaction results in the distortion of the ribosome, misreading of the codon, blockage of initiation of protein synthesis and inhibition of translocation by stabilisation of the binding of peptidyl-tRNA to the ribosomal A site (Russell & Chopra 1990, Kallova *et al* 1995, Vanhoof *et al* 1995).

Macrolide antibiotics are predominantly bacteriostatic and display selective

toxicity by binding to the 50S ribosomal subunit. Erythromycin is an antibiotic which belongs to the macrolide group of antibiotics. Antibiotics which inhibit protein synthesis do so by interfering with ribosomal function (Russell & Chopra 1990). Some macrolide antibiotics inhibit growth in both prokaryotic and eukaryotic cells, whereas others are relatively selective for prokaryotic cells and are therefore, useful antimicrobial agents (Bowman & Rand 1980). Ribosomes from bacteria and blue-green algae can be distinguished from those of higher organisms on the basis of their sedimentation coefficients (S values). The S value (Svedberg units) are determined by the rate at which the ribosomal subunits settle during differential centrifugation. The settlement rate depends upon the molecular weight and shape of the protein molecule and the density of the solvent and the diffusion coefficient of the protein in the solvent. Proteins and other macromolecules have sedimentation coefficients in the range of  $1-200 \times 10^{-13}$  seconds. Thus a protein with a coefficient of  $7 \times 10^{-13}$  seconds would be designated 7S. Prokaryotic cells have 70S ribosomes, which can be readily dissociated into 50S and 30S subunits. Many antibiotics can distinguish between the two types of ribosomes (prokaryotic and eukaryotic) and this accounts for the high degree of selective toxicity that exists within the macrolide and aminoglycoside groups (Bowman & Rand 1980). Erythromycin binds to the larger 50S subunit of the ribosome and prevents the translocation of the tRNA-growing peptide chain complex from the amino acid binding site to the peptidyl site. The antibacterial spectra of all the macrolide antibiotics are essentially similar and are effective against Gram-positive bacteria and some Gram-negative bacteria (Bowman & Rand 1980).

### **Inhibitors of DNA Gyrase**

Nalidixic acid (a 4-quinolone antimicrobial) inhibits DNA gyrase, the enzyme responsible for supercoiling of the bacterial chromosome. The replication of the bacterial chromosome, a circular duplex DNA molecule, requires the separation of the two highly entwined parental strands from one another. During the separation the helix loops are formed (positive supercoiled twists). In order to prevent positive superhelicity, which would result in an increase of torsional strain further preventing unwinding of parental DNA at the replication fork, DNA gyrase relaxes the positively super coiled DNA by breaking a phosphodiester bond in one of the strands of the double helix. DNA gyrase of *E. coli* consists of two gyrase A subunits and two gyrase B subunits. The gyrase A subunits

mediate the transient breakage-rejoining reactions initiated by DNA gyrase. The B subunits are responsible for the introduction of negative supercoils into duplex DNA which has been nicked by the action of subunit A. 4-Quinolones bind predominantly to the A subunits of DNA gyrase resulting in the impairment of both subunits A and B. It impairs the activity of subunit A and the function of subunit B (Russell & Chopra 1990).

### **Inhibitors of Peptidoglycan Synthesis**

Ampicillin (a  $\beta$ -Lactam antibiotic) inhibits the growth of bacteria due to binding of drug molecules to penicillin-binding proteins (PBP'S). The PBP's are located on the surface of bacterial cytoplasmic membrane. PBP's catalyse transpeptidase, carboxypeptidase and endopeptidase reactions. The high molecular weight PBP's (PBP'S 1-3) are responsible for the net synthesis of peptidoglycan *in vivo*. PBP's 1-3 are enzymes which catalyse both the polymerisation of the disaccharide units into glycan chains (transglycosylation) and also the cross-linking of their pentapeptide side chains (transpeptidation) (Vanderwel & Ishiguro 1984, Russell & Chopra 1990). Transpeptidases, in particular are the targets of  $\beta$ -lactam action. The enzyme utilises the D-alanyl-D-alanine moiety of the pentapeptide as a substrate and are believed to form an acyl-enzyme intermediate, accompanied with the release of the terminal D-alanine during the course of the reaction (Kleinhauf & von Dohren 1988, Russell & Chopra 1990). It has been suggested that  $\beta$ -lactams bind covalently to the same group in the enzymes (PBP's), to which the natural substrate normally binds. This binding of  $\beta$ -lactams inactivates transpeptidation thus, resulting in cell death.

### **GROWTH INHIBITORY STUDIES**

When beginning a study on the action of antibiotics, it is important to know or to ascertain the lowest concentration of the antibiotic at which growth of the microorganisms becomes inhibited (minimal inhibitory concentration or MIC). It is also important to understand the effects of the antibiotic upon growth kinetics of the test organisms and the stages in the growth cycle, if any, which are affected by the action of the antibiotic.

The method used to estimate the MIC of antibiotics in this study was the tube

dilution method (Bloomfield 1991). The tube dilution method is well documented and gives a simple and rapid determination of the MIC. Estimation of the MIC by this method utilises the addition of an antibiotic, in increasing concentrations, to sterile media which is then inoculated with a known cell density of the test microorganism. This series of tubes is then incubated for a known period of time (usually 48 hours). After incubation, the MIC is determined by recording the lowest antibiotic concentration exhibiting no visible signs of growth within the tube. It has been suggested that when applied *in vivo* the test antibiotic targets cells which are actively growing. The utilisation of static incubation in this method limits aeration of the incubating cultures and effectively prevents rapid aerobic growth. Although this is a limiting process, it is suggested that it is a more accurate reflection of a natural microbial niche in which cells will be nutrient limited and not exhibiting maximal growth rates (Bloomfield 1991).

### **Estimation of MIC Values**

*Determination of Erythromycin, Ampicillin, Nalidixic acid and Streptomycin MIC's against E. coli and B. subtilis Cultured in Chemically Defined Medium (CDM) using the Tube Dilution Method.*

The MIC's for the four antibiotics of choice were estimated in the CDM particular for each bacteria under observation (page 74). The tube dilution method was used as before, using the MIC estimations obtained from the nutrient broth cultures as the starting concentration for determination of MIC's of the four antibiotics in CDM (Bloomfield 1991). The test for Streptomycin MIC against *E. coli* was determined using final antibiotic concentrations in a total volume of 10mL of 0, 2, 4, 8, 16, 32, 64 and 128 $\mu$ g mL<sup>-1</sup>. The tubes were inoculated and incubated as for the MIC test in nutrient broth and the MIC's were observed and noted (Table 20). The MICs of the antibiotics were all substantially lower in CDM than in nutrient broth, apart from streptomycin against *B. subtilis*. This may be due to streptomycin being less effective under the nutritionally limited conditions when compared to nutrient broth. *B. subtilis* exhibited a slower rate of growth in CDM. The MIC can depend upon the physiology of the bacteria used for the inoculum in the test (Gilbert *et al* 1987). The more slowly the bacteria grow, the slower the rate of killing (Tuomanen *et al* 1986); some compounds become non-bactericidal against slow growing cells (Cozens *et al* 1986). This slower growth rate may result in the *B. subtilis* developing resistance to the streptomycin in

CDM. Therefore, requiring a higher concentration of streptomycin in order to inhibit the bacterial growth. Once the MIC's were determined in CDM, both *E. coli* and *B. subtilis* were grown under the conditions of nutrient limitation and nutrient limitation challenged with sub-MIC concentrations of erythromycin.

**Table 20:** Minimum Inhibitory Concentrations in CDMs.

Antibiotic	<i>E. coli</i>	<i>B. subtilis</i>	Serum concentration (Walker 1993).
Ampicillin	0.5µg mL <sup>-1</sup>	50µg mL <sup>-1</sup>	35.7 - 143µg mL <sup>-1</sup>
Nalidixic acid	1.5µg mL <sup>-1</sup>	4.0µg mL <sup>-1</sup>	142µg mL <sup>-1</sup>
Streptomycin	1.0µg mL <sup>-1</sup>	20µg mL <sup>-1</sup>	143µg mL <sup>-1</sup>
Erythromycin	1.0µg mL <sup>-1</sup>	1.5µg mL <sup>-1</sup>	37.7 - 71.5µg mL <sup>-1</sup>

Erythromycin was the only antibiotic utilised in the following experiments. Erythromycin was chosen due to its action against both Gram-negative and Gram-positive bacteria. It also targets the ribosome, and as the HPN's are ultimately associated with the ribosome, erythromycin may indicate if there is any link between nutrient limitation, nutrient limitation combined with exposure to antibiotics and the production of 'Magic Spot'.

**Determination of ATP Content During the Growth of *E. coli* and *B. subtilis*.**

ATP is the energy-baring molecule of all cells, it belongs to a category of high-energy compounds (including GTP) which liberate energy when the bond between the outermost phosphate (the second and third) is broken. The presence of these “high-energy bonds” makes it possible for ATP to liberate and store energy for cellular chemical reactions. ATP is involved in protein synthesis due to its activation of amino acids, which produces aa-AMP, this group is then attached to the receptor end of tRNA. The accuracy involved in this activation and binding is crucial as the tRNA then converts the mRNA message into a protein

(Talaro & Talaro 1993). Protein synthesis consumes an enormous amount of energy. The equivalent of 3 ATP's is used in forming each peptide bond. One ATP is required to charge the tRNA; one GTP is needed for the binding of the tRNA to the peptidyl (P) site (where the tRNA meets the mRNA) and one more GTP is spent translocating the mRNA from the P site to the aminoacyl (A) site. In total approximately 1,200 ATP's are required to produce one averaged-sized protein (Talaro & Talaro 1993).

The findings of Dinning (1995) suggested that ATP concentration decreased rapidly during the stationary-phase of growth of *Pseudomonas aeruginosa*. The role of ATP in 'Magic Spot' production may be one reason why there is an observed decrease in the concentration of ATP during stationary phase. In order to determine at what point during the growth of the organism and under which particular growth conditions the synthesis of 'Magic Spot' is induced, the concentration of ATP was observed throughout the growth of the culture.

**Effects of Antibiotics on Nutrient Limited Cultures**

*Nutrient Limited Experimental Cultures challenged With Sub-MIC of Erythromycin*

Sterile L&C CDM or M9 media were prepared by the addition of various controlled concentrations of essential nutrients (Tables 21 & 22) and control cultures were prepared for comparison.

**Table 21:** Concentration of nutrients both sufficient (control) and limited (experimental) within M9 medium for the growth of *E. coli* (Koch & Gross 1979).

Component	Concentration	
	Sufficient	Limited
Carbon	2.56x10 <sup>-2</sup> M	2.22x10 <sup>-3</sup> M
Nitrogen	1.87x10 <sup>-3</sup> M	2.0x10 <sup>-4</sup> M



Aliquots (250 & 1000mL) of these media were dispensed into sterile de-ionised 1L and 2L Erlenmyer flasks respectively. These volumes ensured an adequate density of cells was obtained to provide sufficient levels of cellular nucleotide extracts for experimental analysis. The cultures were inoculated with 625 $\mu$ L and 2.5mL respectively, of an overnight control culture of either *B. subtilis* or *E. coli*. Cultures were then grown at 37°C in an orbital incubator (200 osc min<sup>-1</sup>, Gallenkamp INA-305). After 1 hour of growth erythromycin (80% of MIC) was added to each culture. Aliquots (2mL) were removed at 1 hourly intervals after the addition of erythromycin and these samples were spectrophotometrically assayed at E470nm, blanked against a 2mL sample of the appropriate sterile medium. The samples were assayed for ATP concentration according to (pages 67-68). Cell concentrations were determined as CFUmL<sup>-1</sup> at specific given E470nm values (page 62).

**Table 22:** Concentration of nutrients both sufficient (control) and limited (experimental) within L&C CDM for the growth of *B. subtilis* (Leitch & Collier 1996).

Component	Concentration	
	Sufficient	Limited
Carbon (combined glucose & sodium citrate).	2.56x10 <sup>-2</sup> M	1.3x10 <sup>-2</sup> M
Nitrogen	1.87x10 <sup>-3</sup> M	4.8x10 <sup>-5</sup> M

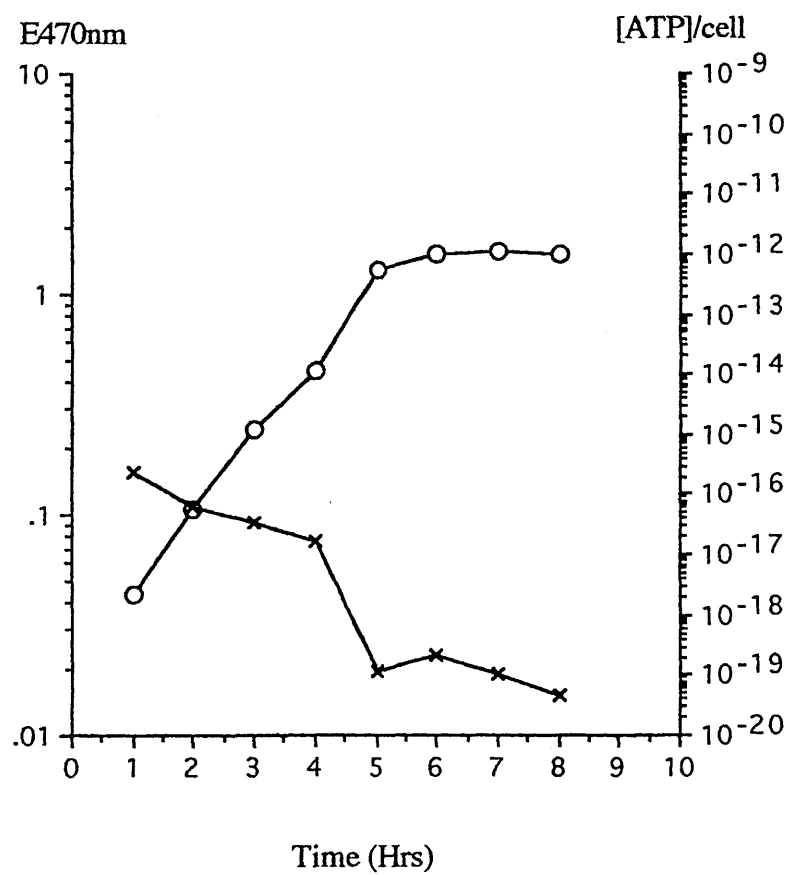
*Determination of ATP Concentration per Cell of Escherichia coli NCIMB 10000 Culture in Carbon and Nitrogen Sufficient M9 Medium*

The control cultures (Carbon,  $2.56 \times 10^{-2} \text{M}$  and Nitrogen,  $1.87 \times 10^{-3} \text{M}$ ) (Fig 26) did not exhibit a lag phase. The culture entered stationary phase after 6 hours of growth. The ATP concentration gradually decreased during the exponential growth phase ( $2.429 \times 10^{-16} \text{M}$  to  $1.61 \times 10^{-17} \text{M}$ ). It was observed that the [ATP] fell sharply one hour before the onset of stationary phase ( $1.61 \times 10^{-17} \text{M}$  to  $1.17 \times 10^{-19} \text{M}$ ). This rapid reduction in ATP concentration per cell may be due to the onset of rapid cell division, in that there was an observed 3-fold increase in cell numbers during this period. The rate of reduction in ATP concentration levelled off after the culture entered stationary phase ( $1.174 \times 10^{-19} \text{M}$  to  $4.399 \times 10^{-20} \text{M}$ ).

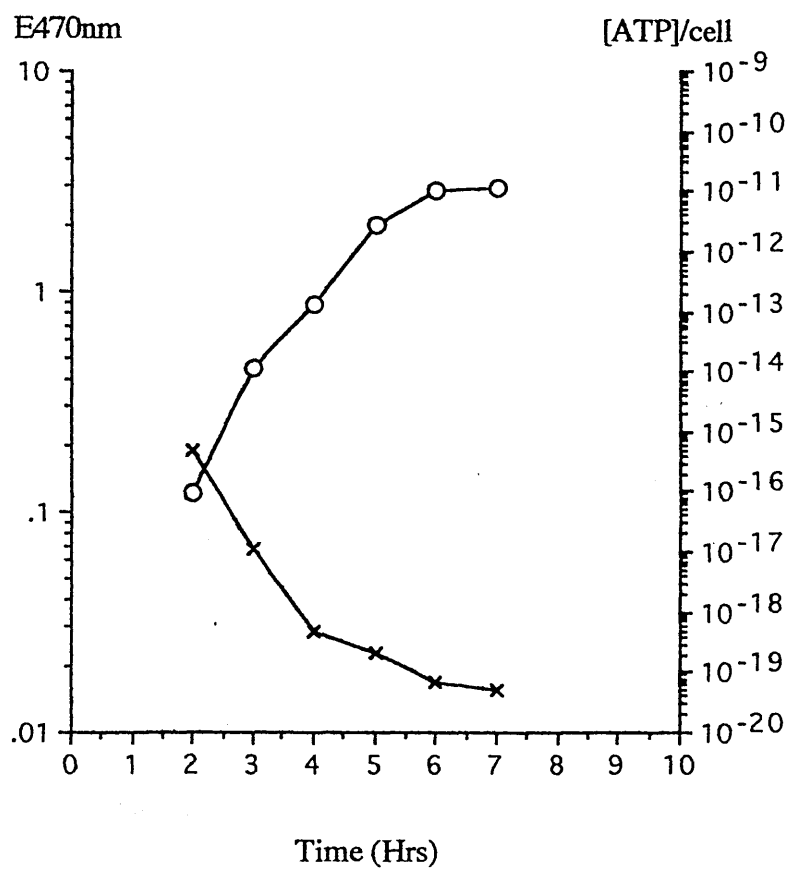
*Determination of ATP Concentration per Cell of Escherichia coli NCIMB 10000 Culture in Carbon and Nitrogen Sufficient M9 Medium challenged with 80% MIC Erythromycin*

The growth of the culture challenged with 80% MIC of erythromycin (Fig 27) was not adversely affected by the presence of the antibiotic. The onset of stationary-phase was not as abrupt as in the nutrient sufficient culture, it was more gradual and occurred after 5 hours, of growth which was 1 hour earlier than the control culture. The concentration of ATP per cell during the growth of *E. coli* under these conditions exhibited decreased from  $4.99 \times 10^{-16} \text{M}$  after 2 hours of growth to  $2.159 \times 10^{-19} \text{M}$  at the onset of stationary-phase. This was due to the cells dividing and actively metabolising thus, utilising the ATP available for growth under these conditions. Once the cells entered stationary phase the ATP concentration per cell continued to decrease, albeit at a reduced rate. The ATP concentration decreased from  $2.159 \times 10^{-19} \text{M}$  at onset of stationary-phase to  $5.09 \times 10^{-20} \text{M}$  after two hours of stationary-phase.

**Figure 26:** Growth (E470nm) (o) of *Escherichia coli* NCIMB 10000 in carbon sufficient ( $2.56 \times 10^{-2} \text{M}$ ) and nitrogen sufficient ( $1.87 \times 10^{-3} \text{M}$ ) M9 medium against (x) ATP concentration per cell (M). For reasons of clarity error bars have been deleted from this figure.



**Figure 27:** Growth (E470nm) (o) of *Escherichia coli* NCIMB 10000 in carbon sufficient ( $2.56 \times 10^{-2} \text{M}$ ) and nitrogen sufficient ( $1.87 \times 10^{-3} \text{M}$ ) M9 medium challenged with  $0.8 \mu\text{g mL}^{-1}$  erythromycin against (x) ATP concentration per cell (M). For reasons of clarity error bars have been deleted from this figure.



*Determination of ATP Concentration per Cell of Escherichia coli NCIMB 10000 Culture in Carbon Limited M9 Medium*

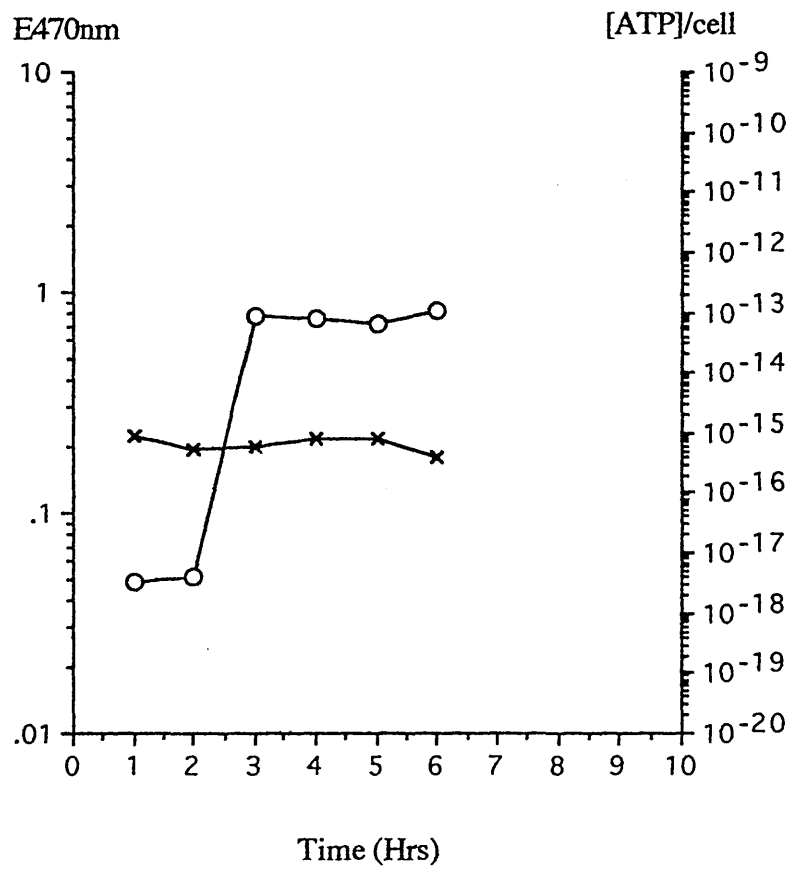
Carbon limited ( $2.22 \times 10^{-3} \text{M}$ ) (Fig 28) cultures exhibited a very short lag phase (1 hour), and the OD (E470nm) increased during the exponential growth phase from 0.052 to 0.78 within one hour. This very short exponential growth phase may have been due to the cells detecting the low levels of carbon available for growth. Thus, they utilised the available carbon in order to attain the maximum growth possible. The ATP concentration per cell during exponential growth phase was  $9.068 \times 10^{-16} \text{M}$  and decreased to  $5.21 \times 10^{-16} \text{M}$ . The decrease in ATP concentration per cell was low when compared to the control culture (the growth attained by both cultures were similar). The ATP concentration in the control culture exhibited a much larger decrease than the ATP concentration in carbon limited conditions. The ATP may have been utilised both cellular metabolism and in the production of 'Magic Spot', as 'Magic Spot' was produced in this culture (this will be discussed later). The culture exhibited an abrupt entry into stationary phase 3 hours after inoculation. The ATP concentration per cell during stationary phase was  $5.77 \times 10^{-16} \text{M}$  and exhibited a decreased to  $4.1 \times 10^{-16} \text{M}$ . This concentration was relatively constant due to the metabolic inactivity of the cells during stationary-phase.

*Determination of ATP Concentration per Cell of Escherichia coli NCIMB 10000 Culture in Carbon Limited M9 Medium challenged with 80% MIC Erythromycin*

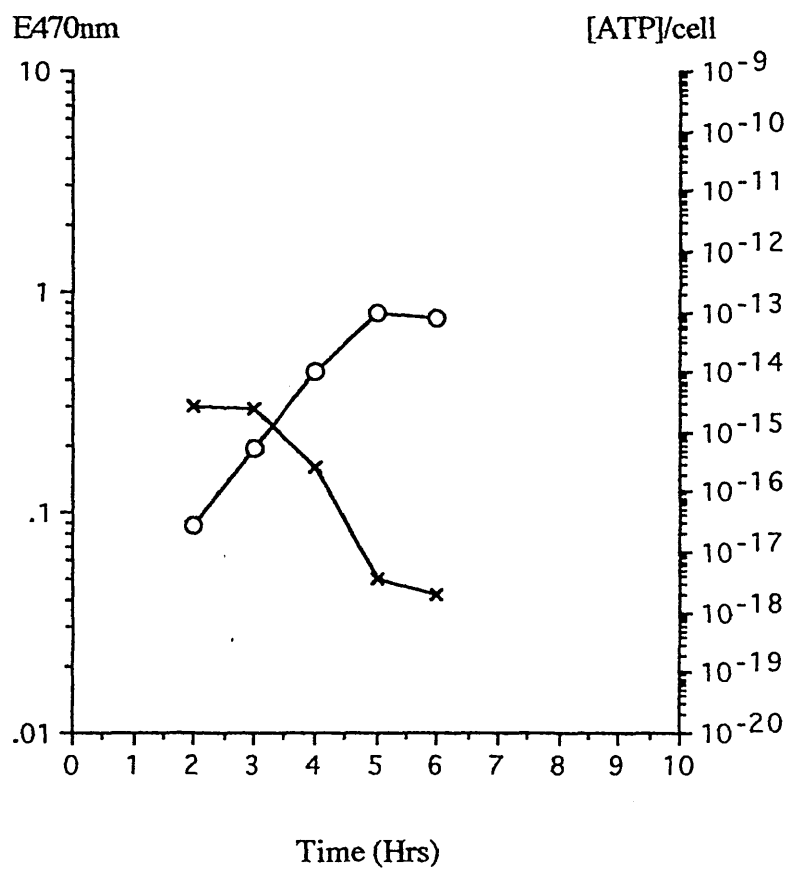
When the carbon limited culture was challenged with  $0.8 \mu\text{g mL}^{-1}$  erythromycin (Fig 29) there was no observed lag phase. *E. coli* often exhibit a very short lag phase and as the cells were left to attenuate to both carbon limitation and exposure to erythromycin, it is possible that the bacteria had exited lag phase and entered exponential growth before the first sample was taken. The optical densities (E470nm) attained in the carbon limited medium challenged with erythromycin (0.087 after 2 hours of growth to 0.75 after 6 hours of growth), were similar to those observed in the carbon limited culture. The challenged culture exhibited a gradual reduction in the concentration of ATP during the exponential growth phase from  $2.709 \times 10^{-15} \text{M}$  per cell after 2 hours of incubation to  $3.524 \times 10^{-18} \text{M}$  at the onset of stationary-phase at 6 hours. As the cells entered stationary-phase the ATP concentration

**Figure 28:** Growth (E470nm) (o) of *Escherichia coli* NCIMB 10000 in carbon limited ( $2.22 \times 10^{-3} \text{M}$ ) M9 medium against (x) ATP concentration per cell (M). For reasons of clarity error bars have been deleted from this figure.





**Figure 29:** Growth (E470nm) (o) of *Escherichia coli* NCIMB 10000 in carbon limited ( $2.22 \times 10^{-3} \text{M}$ ) M9 medium challenged with  $0.8 \mu\text{g mL}^{-1}$  erythromycin against (x) ATP concentration per cell (M). For reasons of clarity error bars have been deleted from this figure.



per cell decreased from  $3.524 \times 10^{-18} \text{M}$  to  $1.953 \times 10^{-18} \text{M}$ .

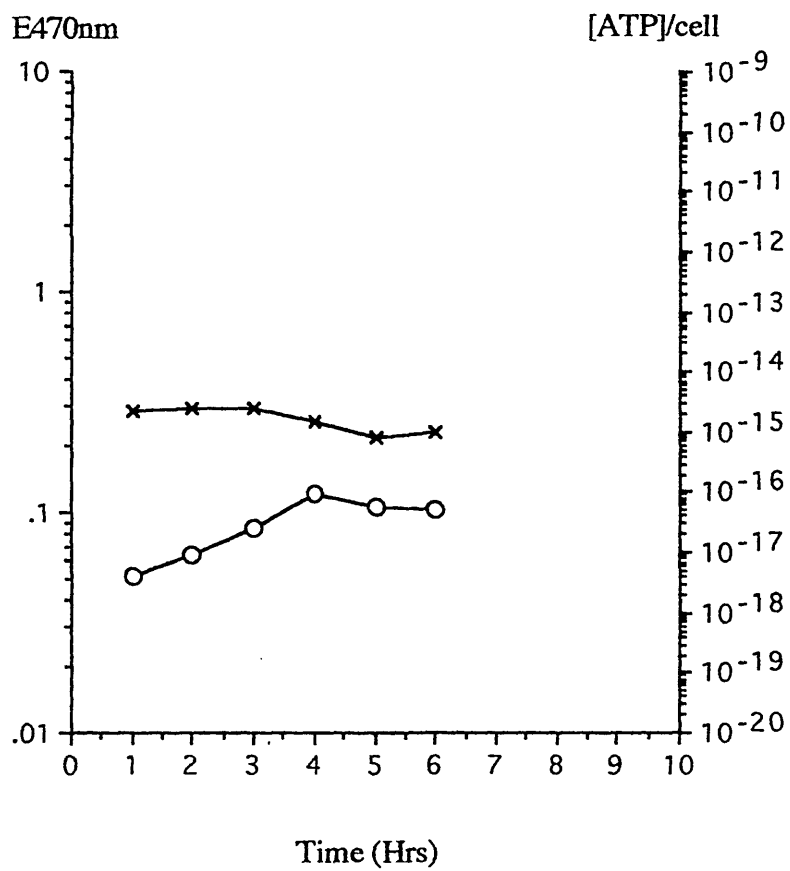
*Determination of ATP Concentration per Cell of Escherichia coli NCIMB 10000 Culture in Nitrogen Limited M9 Medium*

The culture grown under nitrogen limited ( $2.0 \times 10^{-4} \text{M}$ ) (Fig 30) conditions exhibited a 10-fold reduction in cell density when compared to the control culture. There was no observed lag phase, the culture grew for 4 hours after inoculation and attained an OD (E470nm) of 0.121. The ATP concentration per cell decreased from  $2.32 \times 10^{-15} \text{M}$  after 1 hour of incubation to  $1.533 \times 10^{-15} \text{M}$  after 4 hours of growth. The culture entered stationary-phase after 4 hours of incubation. The ATP concentration decreased from  $1.533 \times 10^{-15} \text{M}$  to  $1.02 \times 10^{-15} \text{M}$  during stationary-phase. The overall ATP concentration per cell decreased by half the original concentration. Many bacteria are capable of scavenging nitrogen from other biochemical constituents within the M9 medium and the level of growth observed was probably due to the accessing of an alternative nitrogen source.

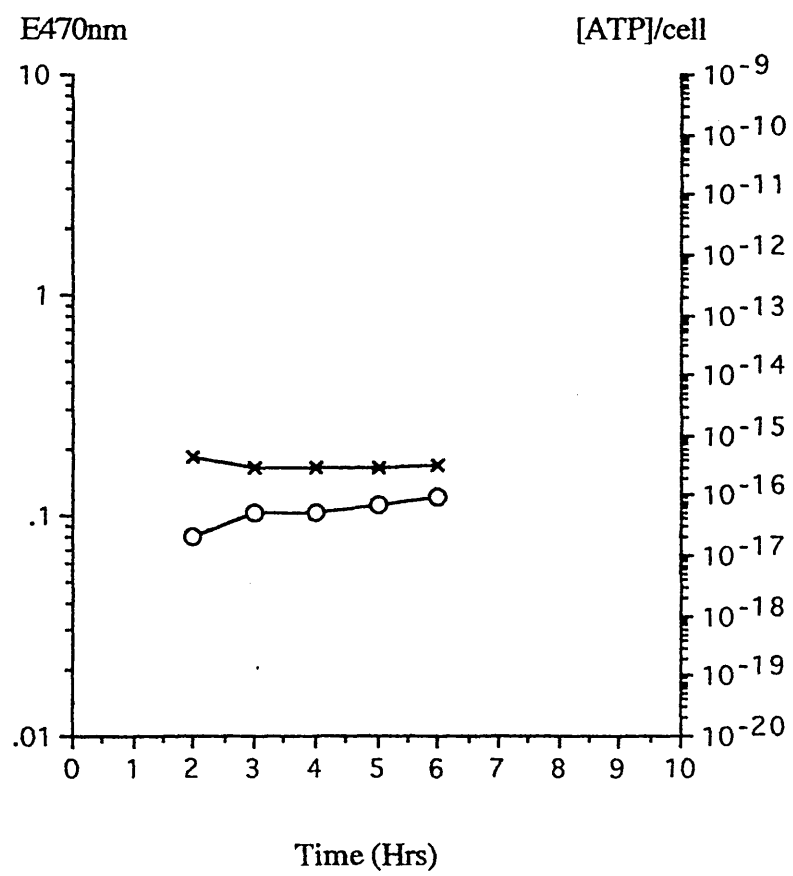
*Determination of ATP Concentration per Cell of Escherichia coli NCIMB 10000 Culture in Nitrogen Limited M9 Medium challenged with 80% MIC Erythromycin*

The culture grown under conditions of nitrogen limitation ( $2.0 \times 10^{-4} \text{M}$ ) challenged with  $0.8 \mu\text{g mL}^{-1}$  erythromycin (Fig 31) exhibited very little growth. The OD (E470nm) increased from 0.081 to a maximum of 0.122, 6 hours after inoculation. The presence of erythromycin did not affect the growth attained by the cells these results were almost identical to those observed in the nitrogen limited culture. There was no distinct entry into stationary-phase. There was, however, an observed reduction in ATP concentration per cell one hour after the erythromycin was added, but this was only a slight reduction from  $4.545 \times 10^{-16} \text{M}$  to  $3.009 \times 10^{-16} \text{M}$ . The ATP concentration was almost constant during the observation of growth and the final recorded concentration was  $3.049 \times 10^{-16} \text{M}$ .

**Figure 30:** Growth (E470nm) (o) of *Escherichia coli* NCIMB 10000 in nitrogen limited ( $2.0 \times 10^{-4} \text{M}$ ) M9 medium against (x) ATP concentration per cell (M). For reasons of clarity error bars have been deleted from this figure.



**Figure 31:** Growth (E470nm) (o) of *Escherichia coli* NCIMB 10000 in nitrogen limited ( $2.0 \times 10^{-4} \text{M}$ ) M9 medium challenged with  $0.8 \mu\text{g mL}^{-1}$  erythromycin against (x) ATP concentration per cell (M). For reasons of clarity error bars have been deleted from this figure.





*Determination of ATP Concentration per Cell of **Bacillus subtilis** NCIMB 12900 Culture in Carbon and Nitrogen Sufficient L&C CDM*

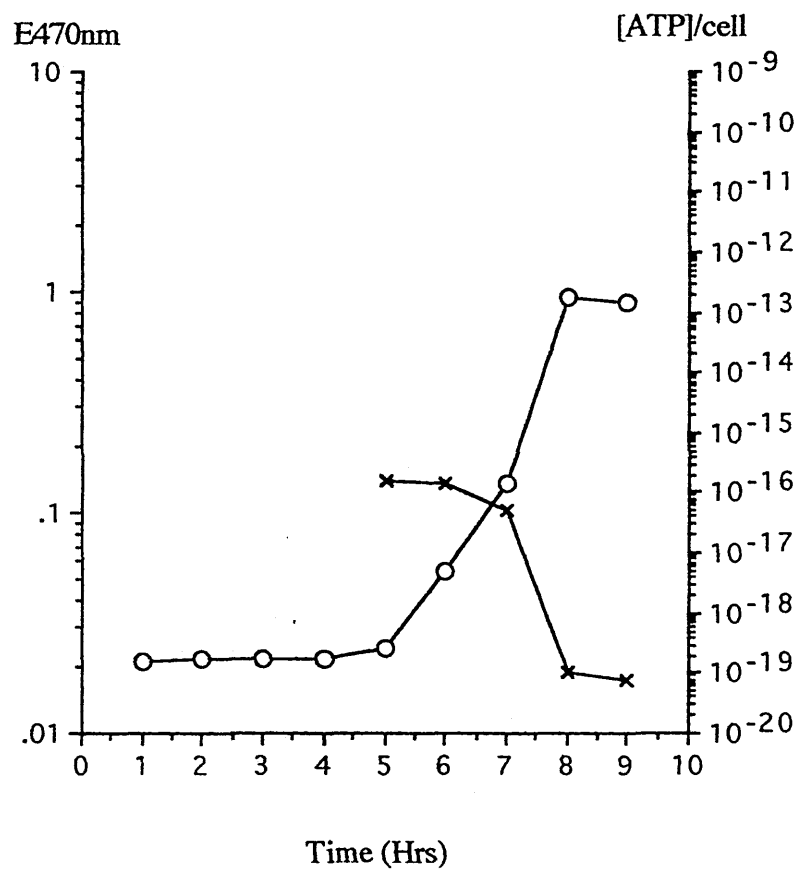
The control cultures (Carbon,  $2.56 \times 10^{-2} \text{M}$  and Nitrogen,  $1.87 \times 10^{-3} \text{M}$ ) (Fig 32) cultures of *B. subtilis* exhibited a lag phase of 5 hours and an exponential growth phase of 3 hours. The onset of stationary phase was abrupt and the OD of the cells fell to 0.904 once the culture was in stationary phase.

During lag phase there was no detectable ATP, this was possibly due to the cells preparing for cell division thus, the ATP present within the cells being utilised for growth and metabolism. As the cells began to grow and divide during the exponential growth phase, ATP was detected. The concentration per cell of ATP at 5 hours was  $1.526 \times 10^{-16} \text{M}$ , this decreased to  $9.975 \times 10^{-20} \text{M}$  at 8 hours. The reduction in the concentration of ATP per cell was due to normal growth and division during the exponential growth phase. Once the cells entered stationary phase the ATP concentration per cell continued to decrease, albeit it at a slower rate. There was no production of 'Magic Spot' under these conditions and the ATP concentration remained relatively constant.

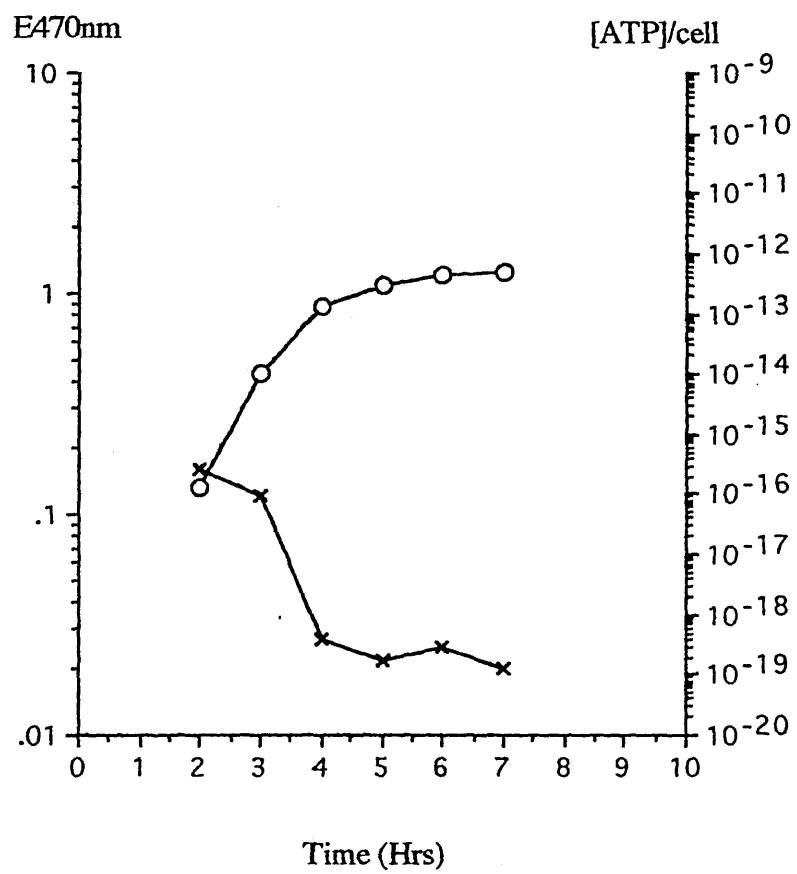
*Determination of ATP Concentration per Cell of **Bacillus subtilis** NCIMB 12900 Culture in Carbon and Nitrogen Sufficient L&C CDM challenged with 80% MIC Erythromycin.*

The control culture (Carbon,  $2.56 \times 10^{-2} \text{M}$  and Nitrogen,  $1.87 \times 10^{-3} \text{M}$ ) (Fig 33) challenged with  $1.2 \mu\text{gmL}^{-1}$  erythromycin did not exhibit a lag phase. The culture was in exponential growth phase 2 hours after inoculation. This culture attained a higher cell density in a shorter period of time than the carbon and nitrogen sufficient culture unchallenged with erythromycin. The OD (E470nm) at 2 hours was 0.131 and increased to 1.094 at 5 hours. The cells entered stationary-phase after five hours of growth and the OD (E470nm) at 7 hours was 1.244. The ATP concentration per cell steadily decreased from  $2.637 \times 10^{-16} \text{M}$  to  $1.645 \times 10^{-19} \text{M}$  at the onset of stationary phase. During stationary phase the ATP concentration per cell remained fairly constant, after 7 hours the ATP concentration per cell was  $1.265 \times 10^{-19} \text{M}$ .

**Figure 32:** Growth (E470nm) (o) of *Bacillus subtilis* NCIMB 12900 in carbon sufficient ( $2.56 \times 10^{-2} \text{M}$ ) and nitrogen sufficient ( $1.87 \times 10^{-3} \text{M}$ ) L&C CDM against (x) ATP concentration per cell (M). For reasons of clarity error bars have been deleted from this figure.



**Figure 33:** Growth (E470nm) (o) of *Bacillus subtilis* NCIMB 12900 in carbon sufficient ( $2.56 \times 10^{-2} \text{M}$ ) and nitrogen sufficient ( $1.87 \times 10^{-3} \text{M}$ ) L&C CDM challenged with  $1.2 \mu\text{g mL}^{-1}$  erythromycin against (x) ATP concentration per cell (M). For reasons of clarity error bars have been deleted from this figure.



*Determination of ATP Concentration per Cell of Bacillus subtilis NCIMB 12900 Culture in Carbon Limited L&C CDM*

The cultures of *B. subtilis* grown under carbon limited ( $1.3 \times 10^{-4} \text{M}$ ) (Fig 34) conditions exhibited a short lag phase of 1 hour. During lag phase the ATP concentration remained relatively constant at  $4.559 \times 10^{-15} \text{M}$  to  $4.265 \times 10^{-15} \text{M}$ . This was probably due to the cells attenuating themselves to the carbon limited conditions. During the exponential growth phase the OD (E470nm) increased from 0.031 to 1.63 after 8 hours of growth. Once exponential growth commenced, the ATP concentration began to fall. This decrease was very gradual until mid-exponential phase. There was an observed decrease in ATP concentration, which fell from  $4.479 \times 10^{-15} \text{M}$  to  $4.53 \times 10^{-18} \text{M}$  between 5 and 6 hours (the onset of stationary phase). During stationary phase the ATP concentration decreased from  $5.5 \times 10^{-19} \text{M}$  to  $8.766 \times 10^{-19} \text{M}$  at 8 hours. It is possible that the continued decrease in ATP concentration during stationary-phase is due to the production of 'Magic Spot'.

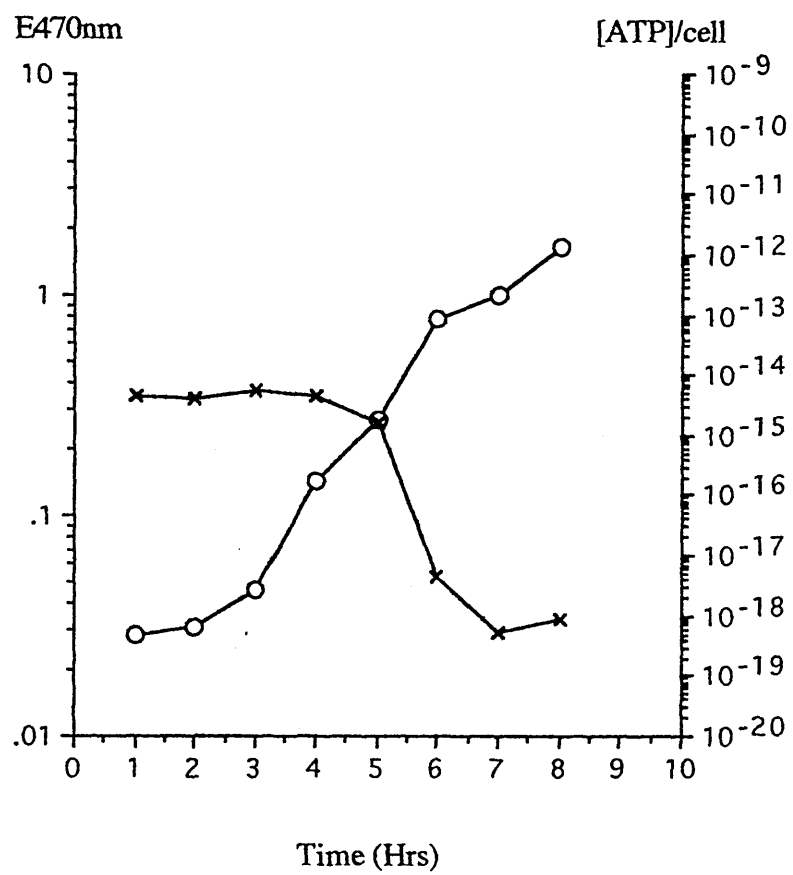
*Determination of ATP Concentration per Cell of Bacillus subtilis NCIMB 12900 Culture in Carbon Limited L&C CDM challenged with 80% MIC Erythromycin.*

The carbon limited culture challenged with  $1.2 \mu\text{g mL}^{-1}$  erythromycin (Fig 35) exhibited no growth. The concentration of ATP per cell exhibited a small, but gradual increase between 3 and 6 hours of incubation, the concentrations were  $2.504 \times 10^{-15} \text{M}$  and  $2.244 \times 10^{-14} \text{M}$  respectively. The final concentration was  $1.42 \times 10^{-14} \text{M}$  during the final 3 hours of the experiment. It would appear that sub-MIC erythromycin inhibited the growth of the culture, as the cell density attained was substantially lower than both the control and the carbon limited culture. Erythromycin inhibits protein synthesis and combined with the nutrient limitation the cells failed to achieve any growth.

*Determination of ATP Concentration per Cell of Bacillus subtilis NCIMB 12900 Culture in Nitrogen Limited L&C CDM*

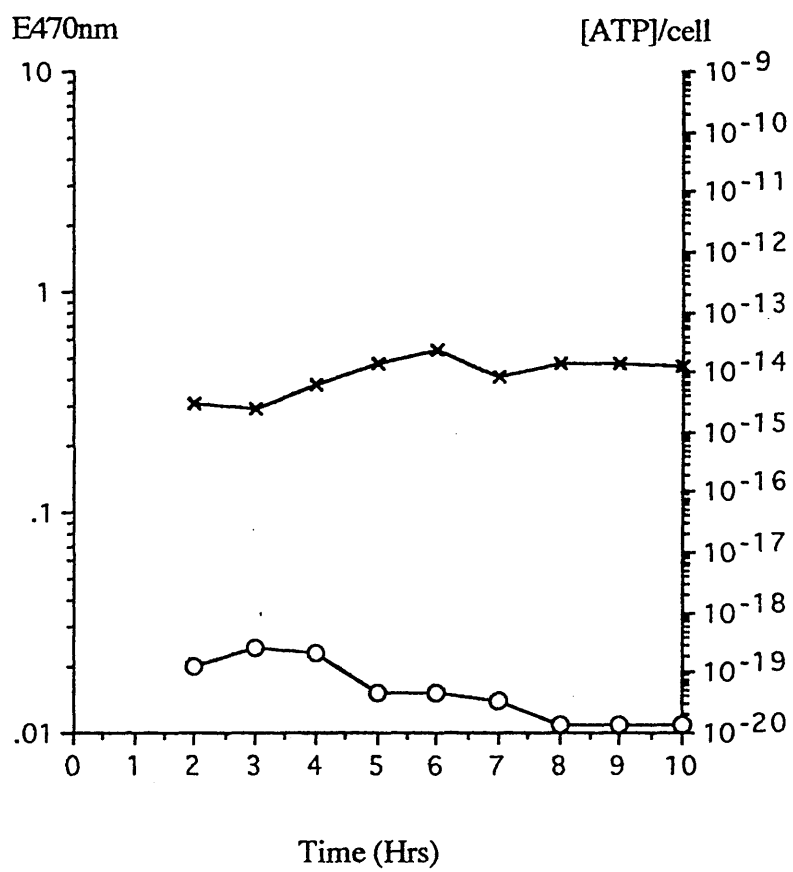
*B. subtilis* exposed to nitrogen limitation ( $4.8 \times 10^{-6} \text{M}$ ) (Fig 36) displayed a lag phase of 3 hours. During the exponential growth phase the OD (E470nm) increased from 0.021 to 1.75 at 6 hours. This level of growth was higher than that observed in the control

**Figure 34:** Growth (E470nm) (o) of *Bacillus subtilis* NCIMB 12900 in carbon limited ( $1.3 \times 10^{-4} \text{M}$ ) L&C CDM against (x) ATP concentration per cell (M). For reasons of clarity error bars have been deleted from this figure.





**Figure 35:** Growth (E470nm) (o) of *Bacillus subtilis* NCIMB 12900 in carbon limited ( $1.3 \times 10^{-4} \text{M}$ ) challenged with  $1.2 \text{gmL}^{-1}$  erythromycin L&C CDM against (x) ATP concentration per cell (M). For reasons of clarity error bars have been deleted from this figure.



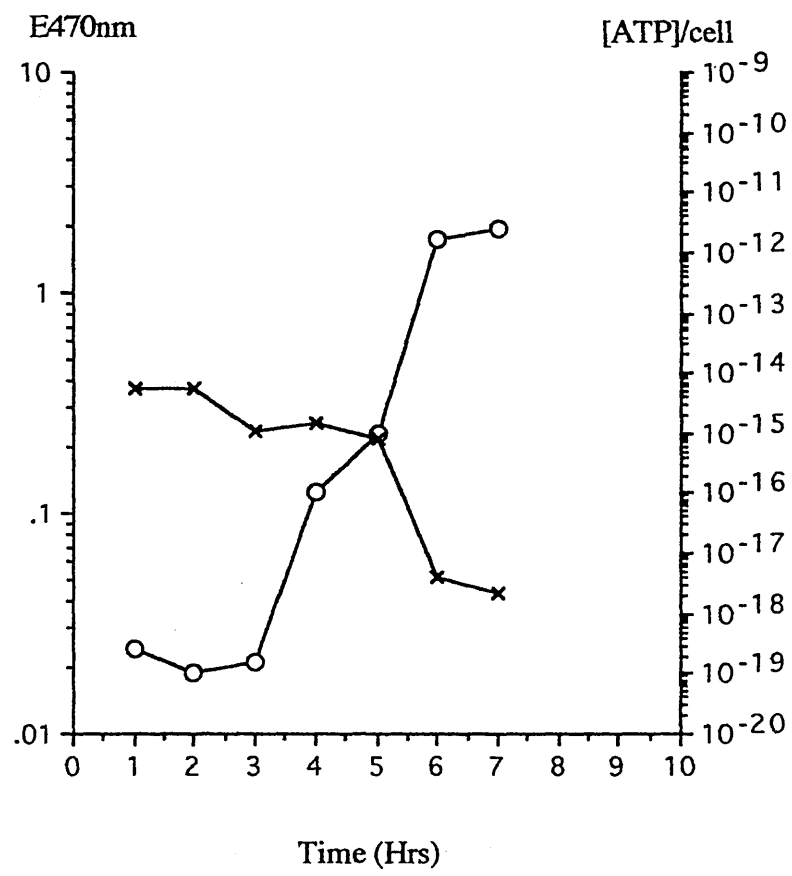
culture. This high level of cell density was possibly due to cells scavenging nitrogen from the other chemical constituents present in the L&C CDM in order to attain the maximum growth possible. The ATP concentration per cell was observed to be inverse to the growth curve. It was constant for the first two hours of incubation at  $5.45 \times 10^{-15} \text{M}$ . During the exponential growth phase it decreased gradually from  $5.454 \times 10^{-15} \text{M}$  to  $7.976 \times 10^{-16} \text{M}$  at 5 hours. The final hour of exponential growth phase exhibited an decrease in ATP concentration from  $7.976 \times 10^{-16} \text{M}$  to  $3.875 \times 10^{-18} \text{M}$ . During stationary phase the ATP concentration decreased from  $3.875 \times 10^{-18} \text{M}$  to  $2.135 \times 10^{-18} \text{M}$ .

*Determination of ATP Concentration per Cell of Bacillus subtilis NCIMB 12900 Culture in Nitrogen Limited L&C CDM challenged with 80% MIC Erythromycin.*

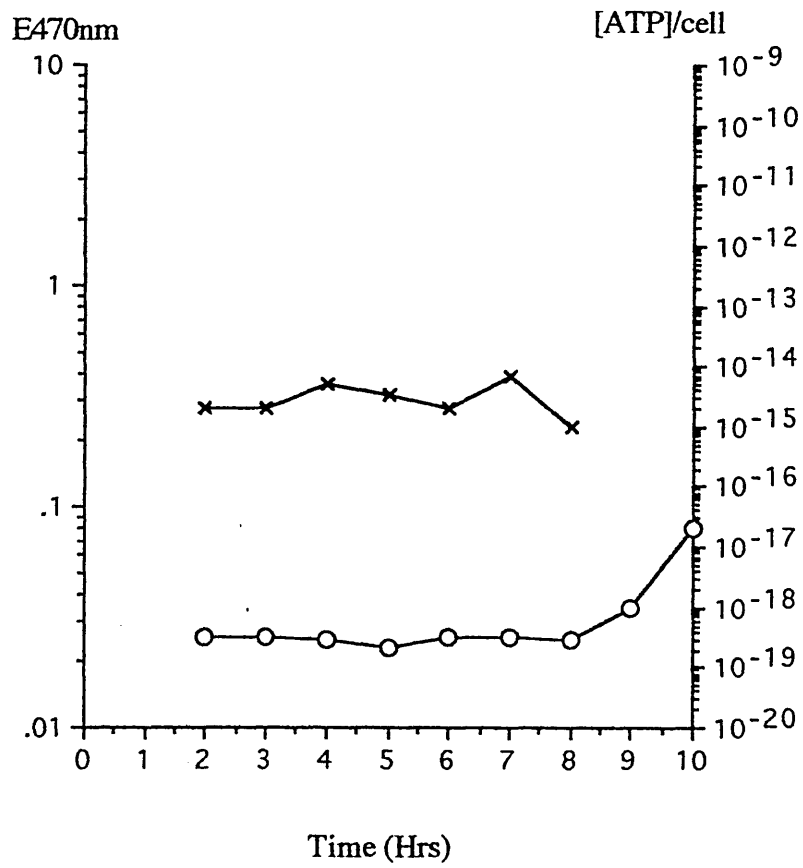
The nitrogen limited culture ( $4.8 \times 10^{-6} \text{M}$ ) challenged with  $1.2 \mu\text{gmL}^{-1}$  erythromycin (Fig 37) exhibited very little growth. The lag phase was 8 hours in duration followed by a brief exponential phase. As with the carbon limited culture challenged with erythromycin, the erythromycin appeared to inhibit the growth of the cells due to the combination of both nutrient limitation and presence of sub-MIC erythromycin.

The ATP concentration per cell increased slightly as the cell number decreased. The concentration of ATP per cell after 2 hours of incubation was  $1.972 \times 10^{-15} \text{M}$ , which increased to  $5.128 \times 10^{-15} \text{M}$  after 4 hours of incubation. It then gradually decreased to  $1.972 \times 10^{-15} \text{M}$  at 6 hours. During the observed period the ATP concentration decreased from  $1.972 \times 10^{-15} \text{M}$  to  $1.02 \times 10^{-15} \text{M}$ . During the final hour of analysis there was no detectable ATP present within the cells.

**Figure 36:** Growth (E470nm) (o) of *Bacillus subtilis* NCIMB 12900 in nitrogen limited ( $4.8 \times 10^{-6} \text{M}$ ) L&C CDM against (x) ATP concentration per cell (M). For reasons of clarity error bars have been deleted from this figure.



**Figure 37:** Growth (E470nm) (o) of *Bacillus subtilis* NCIMB 12900 in nitrogen limited ( $4.8 \times 10^{-6} \text{M}$ ) L&C CDM challenged with  $1.2 \mu\text{g mL}^{-1}$  erythromycin against (x) ATP concentration per cell (M). For reasons of clarity error bars have been deleted from this figure.



## High Performance Liquid Chromatography Analysis of Bacterial Culture Extracts Exposed to Nutrient Limitation and Challenged with sub-MIC Erythromycin.

The isocratic HPLC system was established and recalibrated with standard nucleotides (Table 23).

**Table 23:** Retention times of nucleotides and HPN's  
using an isocratic HPLC system

Nucleotides (20 $\mu$ L of 5x10 <sup>-5</sup> mg)	R <sub>t</sub> values(mins)
AMP	2.3
GMP	2.0
ADP	2.4
GDP	2.6
ATP	3.1
GTP	4.1
MSI	3.8
MSII	2.5

An elution time of 10 minutes was used at a flow rate of 1.5mL per minute, as this was the length of time the high ionic strength buffer required for the elution of HPNs in the previously used gradient HPLC system of Ochi *et al* (1981). After calibration of the column, the control HPNs, produced by enzymic culture filtrate of *S. adepospholyticus* were analysed. Aliquots (20 $\mu$ L) of concentrated fractions 35 and 41 (collected from FPLC analysis of *S. adepospholyticus* culture filtrate) were assayed using the new isocratic HPLC method developed by Leitch and Collier. Aliquots (20 $\mu$ L) of nucleotides (AMP,

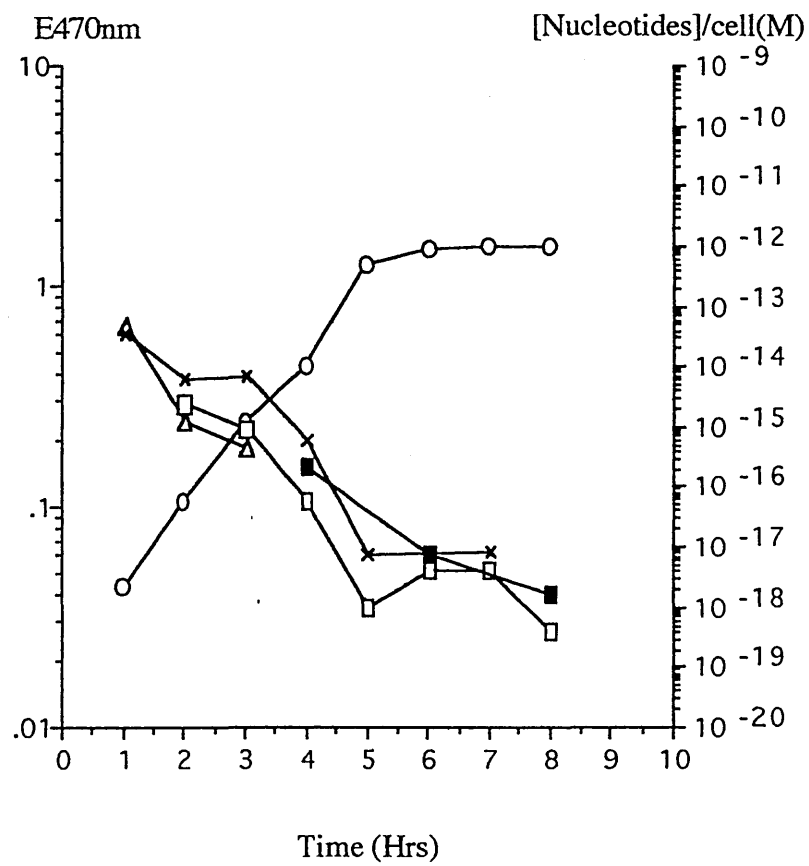


GMP, ADP, GDP, ATP or GTP at concentrations of  $1 \times 10^{-5}$  mg in 0.27M LiCl) were assayed using the new isocratic method. The retention time ( $R_t$ ) for the each HPN and standard nucleotide was determined (Table 23) thus, enabling the analysis of the cellular nucleotide extracts which had been collected from *E. coli* and *B. subtilis* grown under both nutrient limited and nutrient limited antibiotic challenged conditions. These bacterial extracts were stored at  $-70^\circ\text{C}$  until required.

*Determination of the Concentration of Nucleotides per Cell of Escherichia coli NCIMB 10000 Culture in Carbon and Nitrogen Sufficient M9 Medium*

The control cultures (Carbon,  $2.56 \times 10^{-2}$  M and Nitrogen,  $1.87 \times 10^{-3}$  M sufficient) (Fig 38) exhibited a reduction in the concentration per cell of all the observed cellular nucleotides (ie. AMP, GMP, GDP and ATP) during the exponential phase. This was due to the cell growing and metabolising thus, the nucleotides were being utilised for the cellular metabolic requirements. AMP was detected during the first three hours of exponential growth of *E. coli*, and was observed to decrease gradually. It decreased from  $4.0816 \times 10^{-14}$  M after one hour of incubation to  $4.49944 \times 10^{-16}$  M after 3 hours. The presence of AMP coincided with a small decrease in concentration of ATP,  $3.0612 \times 10^{-14}$  M after one hour of incubation to  $6.18673 \times 10^{-15}$  M after 3 hours. Once ATP began to decrease in concentration no AMP was detected. It was thought that this was due to the AMP being used for ATP biosynthesis. No GTP was detected within the cell extracts. This was possibly due to the intracellular GTP being bound up in protein synthesis in order to sustain the metabolic demands of the growing culture. However, as the culture entered stationary phase the concentration of nucleotides per cell decreased at a slightly slower rate than that observed during the exponential phase. This was probably the result of the cellular metabolism slowing down and the quiescence of cellular division.

**Figure 38:** Growth (E470nm) (o) of *Escherichia coli* NCIMB 10000 in carbon ( $2.56 \times 10^{-2} \text{M}$ ) and nitrogen ( $1.87 \times 10^{-3} \text{M}$ ) sufficient M9 medium against ( $\Delta$ ) AMP; ( $\square$ ) GMP; ( $\blacksquare$ ) GDP; ( $\times$ ) ATP concentration per cell (M). For reasons of clarity error bars have been deleted from this figure.



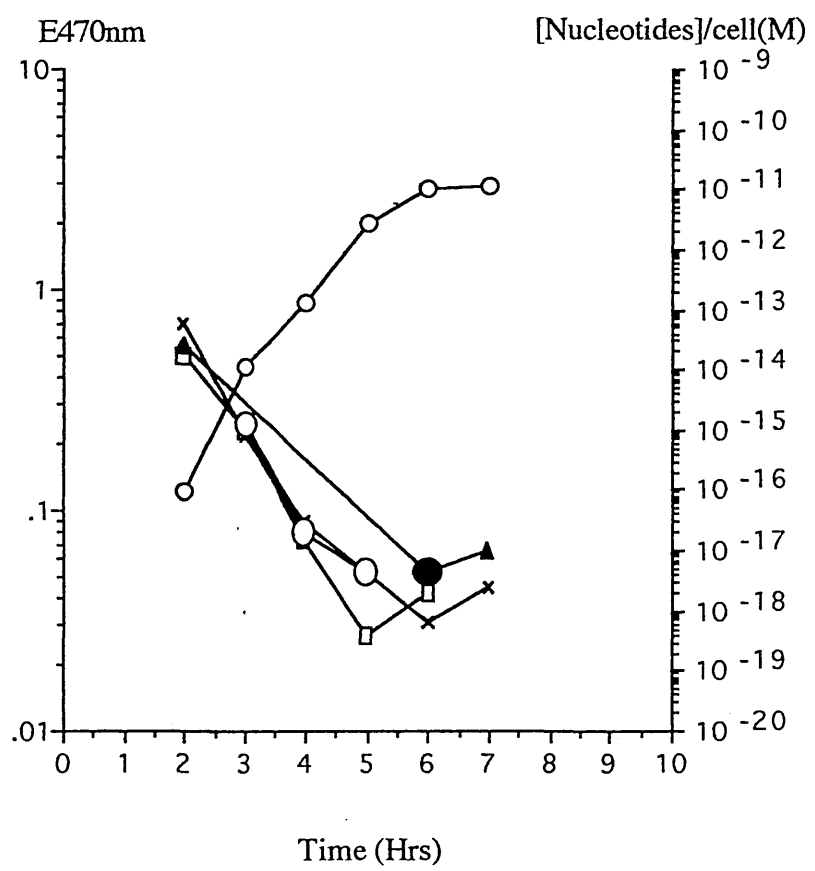
*Determination of the Concentration of Nucleotides per Cell of Escherichia coli NCIMB 10000 Culture in Carbon and Nitrogen Sufficient M9 Medium challenged with 80% MIC Erythromycin.*

The observation of the concentration of cellular nucleotides of the *E. coli* cultures in (Carbon,  $2.56 \times 10^{-2} \text{M}$  and Nitrogen,  $1.87 \times 10^{-3} \text{M}$  sufficient) M9 medium challenged with  $0.8 \mu\text{gmL}^{-1}$  erythromycin (Fig 39) exhibited a much sharper reduction during exponential phase in the presence of erythromycin. As the nucleotides decreased the HPN, ppGpp was detected after 3 hours of incubation (during the exponential phase) and was present until the onset of stationary phase at 5 hours. Once the cells had entered stationary phase pppGpp was detected at one point only (6 hours). The detection of both ppGpp and pppGpp coincided with a rapid decrease in ATP concentration throughout the exponential growth of the culture. The concentration of ATP per cell continued to decrease once the cells had entered stationary phase, but once 'Magic Spot' was no longer detectable the concentration of ATP increased. The increase in the concentration of ATP was probably due to the cells ceasing production of 'Magic Spot' once in stationary phase and therefore, liberating ATP. There was no detectable GTP within the cell extracts of this culture. GTP may have been utilised in both protein synthesis, as the cell density attained was comparable with the control culture, and production of ppGpp and pppGpp. Erythromycin stimulates the dissociation of peptidyl-tRNA from the ribosomes during translocation. If this occurred, the GTP present within the cell would be converted to ppGpp and pppGpp as it could not be utilised in protein synthesis. The presence of erythromycin appeared to be responsible for inducing the production of both ppGpp and pppGpp, as these compounds were not detected in the control culture. The presence of erythromycin may also be responsible for the rapid reduction in the nucleotide concentration observed within the cell during exponential phase.

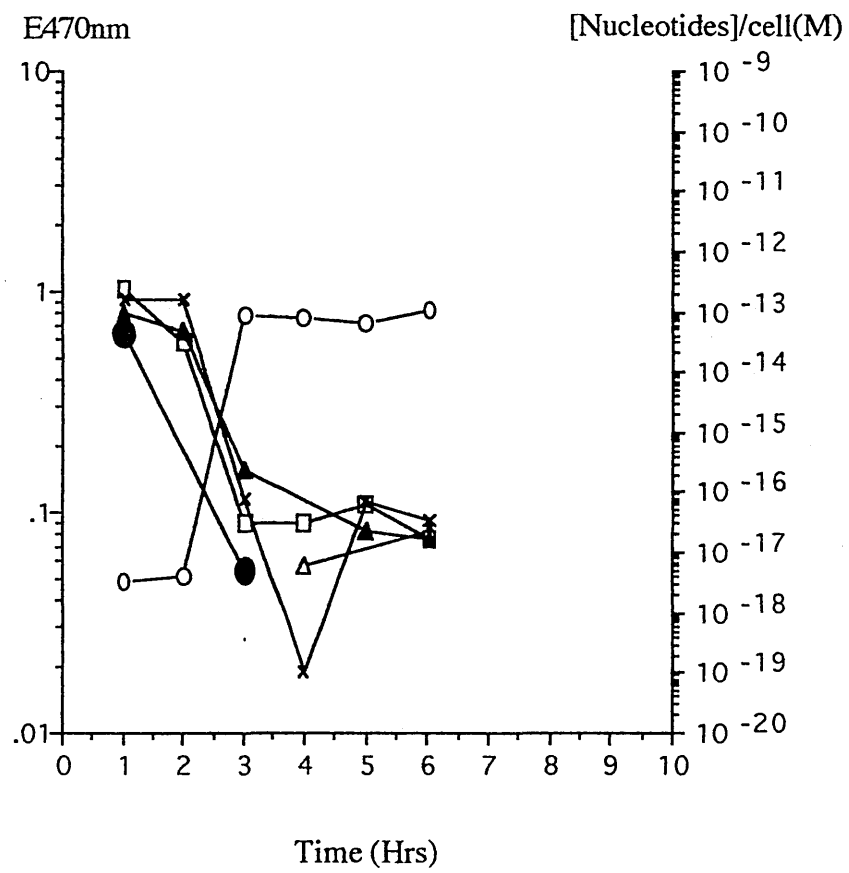
*Determination of the Concentration of Nucleotides per Cell of Escherichia coli NCIMB 10000 Culture in Carbon Limited M9 Medium*

*E. coli* grown in carbon limited ( $2.22 \times 10^{-3} \text{M}$ ) M9 medium (Fig 40) exhibited a decrease in the concentration of nucleotides per cell during exponential growth phase of the culture. Once the culture entered stationary phase the nucleotide concentrations of GMP and ADP remained relatively constant. ATP concentration decreased during lag phase and

**Figure 39:** Growth (E470nm) (o) of *Escherichia coli* NCIMB 10000 in carbon (2.56x10<sup>-2</sup>M) and nitrogen (1.87x10<sup>-3</sup>M) sufficient M9 medium challenged with 0.8μgmL<sup>-1</sup> erythromycin against (□) GMP; (▲) ADP; (×) ATP; (○)ppGpp; (●) pppGpp; concentration per cell (M). For reasons of clarity error bars have been deleted from this figure.



**Figure 40:** Growth (E470nm) (o) of *Escherichia coli* NCIMB 10000 in carbon limited ( $2.22 \times 10^{-3} \text{M}$ ) M9 medium against ( $\Delta$ ) AMP; ( $\square$ ) GMP; ( $\blacktriangle$ ) ADP; ( $\times$ ) ATP; ( $\bullet$ ) pppGpp; concentration per cell (M). For reasons of clarity error bars have been deleted from this figure.





exponential growth phase until the cells had entered stationary phase. It then increased sharply. AMP appeared at 4 hours (stationary phase) and coincides with an increase in ATP concentration. As AMP is involved in ATP synthesis and this synthesis is apparently inhibited, this could explain why AMP was detected only from this time onwards. The decrease in concentration per cell of ATP was accompanied with the production of pppGpp, ('Magic Spot' II). pppGpp was detected for the first three hours of incubation and disappeared at the onset of stationary phase. After its disappearance, the concentration of ATP increased to approximately the same level as the other cellular nucleotides. The nucleotide GTP was not detected and this may have been due to the production of 'Magic Spot'.

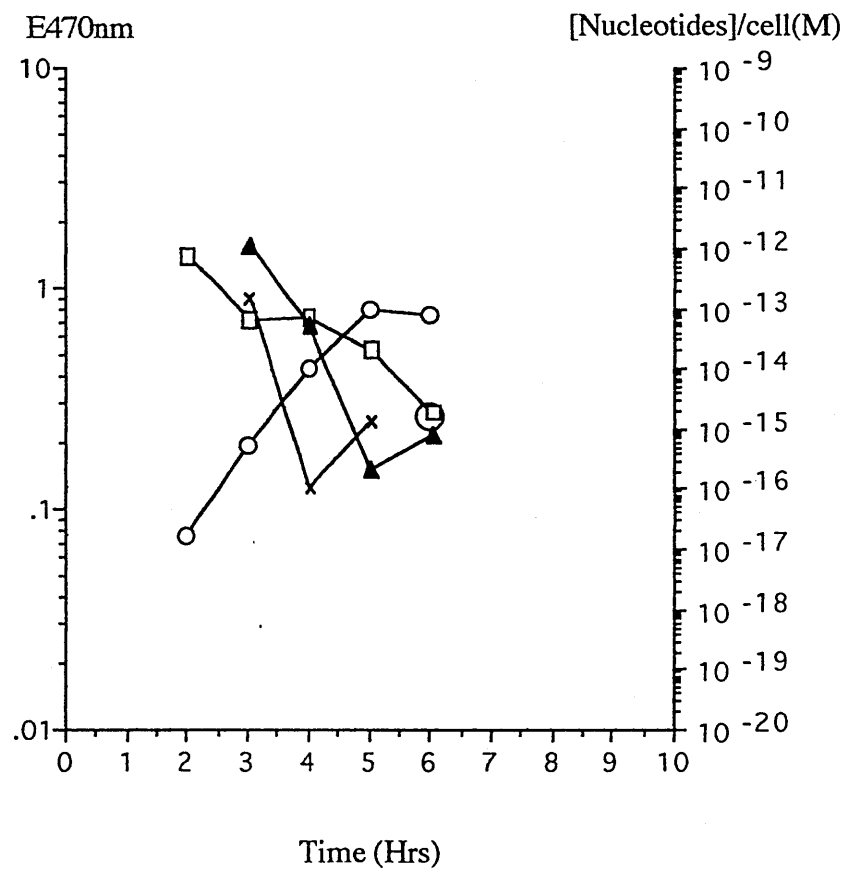
*Determination of the Concentration of Nucleotides per Cell of Escherichia coli NCIMB 10000 Culture in Carbon Limited M9 Medium challenged with 80% MIC Erythromycin.*

*E. coli* grown in carbon limited ( $2.22 \times 10^{-3} \text{M}$ ) M9 medium challenged with  $0.8 \mu\text{g mL}^{-1}$  erythromycin (Fig 41) exhibited the presence of fewer nucleotides than the control cultures. The concentration of the nucleotides per cell decreased throughout the exponential growth phase. ATP and ADP were not detected until the culture had been incubated for three hours and their concentrations decreased in parallel to one another. The ADP detected may be the by-product of the enzymatic cleavage of a phosphate molecule from ATP during the production of 'Magic Spot' thus, liberating ADP. At the onset of stationary phase the ATP concentration increased, this was possibly due to the cell density becoming constant, and the free ATP not being consumed for energy and metabolism. Once the cells entered stationary phase there was no detectable ATP, but there was an increase in ADP concentration. The disappearance of ATP and increase in ADP may be explained by the appearance of pppGpp one hour later and the ATP being utilised in its production and the liberation of ADP as the by-product of this enzymatic cleavage.

*Determination of the Concentration of Nucleotides per Cell of Escherichia coli NCIMB 10000 Culture in Nitrogen Limited M9 Medium*

During growth in nitrogen limited ( $2.0 \times 10^{-4} \text{M}$ ) M9 medium there was very little growth attained within the culture and the decrease in nucleotide concentration per cell was

**Figure 41:** Growth (E470nm) (o) of *Escherichia coli* NCIMB 10000 in carbon (2.22x10<sup>-3</sup>M) limited M9 medium challenged with 0.8μgmL<sup>-1</sup> erythromycin against (□) GMP; (▲) ADP; (×) ATP; (○) ppGpp; concentration per cell (M). For reasons of clarity error bars have been deleted from this figure.



relatively small in comparison to the control culture (data not shown). ATP concentrations decreased as the cells grew and divided and there was a sharp reduction in its presence once the cells had entered stationary phase. The overall concentration of ATP per cell was lower than the control and carbon limited cultures, both with and without the presence of erythromycin. There was no production of 'Magic Spot' under nitrogen limited conditions. Although the growth under conditions of nitrogen limitation was low, there was an increase in cell density, which may be due to *E. coli*'s ability to scavenge nitrogen from other chemical sources within the M9 limited medium.

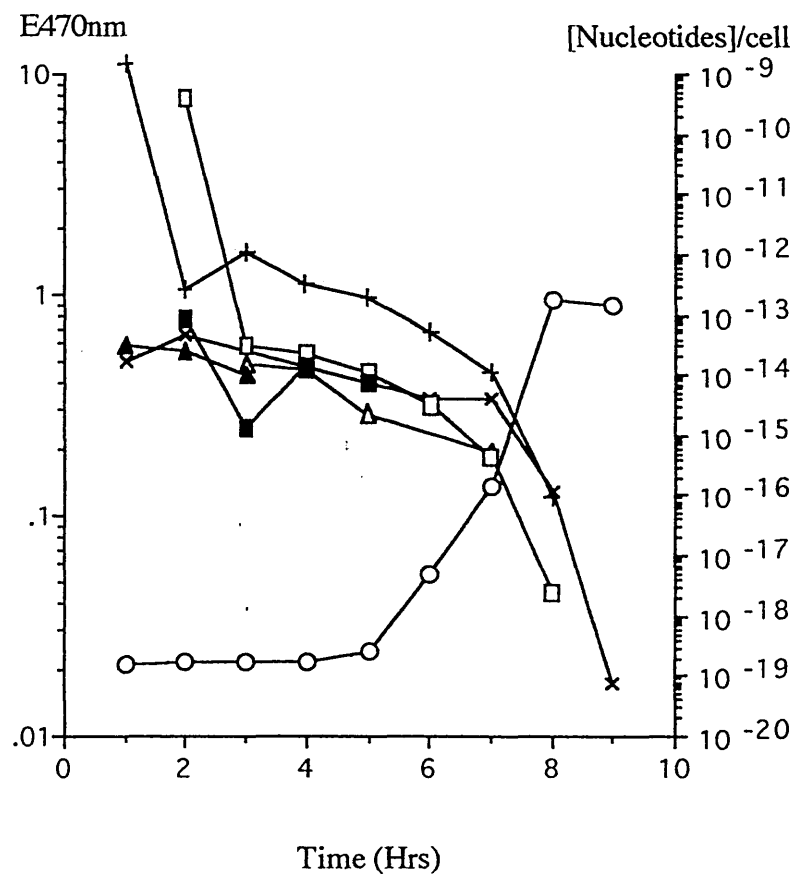
*Determination of the Concentration of Nucleotides per Cell of Escherichia coli NCIMB 10000 Culture in Nitrogen Limited M9 Medium challenged with 80% MIC Erythromycin*

The presence of erythromycin ( $0.8\mu\text{g mL}^{-1}$ ), did not affect the overall growth of the nitrogen limited ( $2.0 \times 10^{-4}\text{M}$ ) culture when compared to nitrogen limitation alone (data not shown). The cell density attained here was comparable to the nitrogen limited culture. There was no significant decrease in ATP concentration during the observed growth of the culture. One hour after the addition of erythromycin to the culture, the concentration per cell of the cellular nucleotides AMP, GMP and GDP all began to decrease. The combined stress of nitrogen limitation and the presence of erythromycin inhibited the growth of *E. coli*.

*Determination of the Nucleotide Concentration per Cell of Bacillus subtilis NCIMB 12900 Culture in Carbon and Nitrogen Sufficient L&C CDM*

The control culture carbon ( $2.56 \times 10^{-2}\text{M}$ ) and nitrogen ( $1.87 \times 10^{-3}\text{M}$ ) sufficient (Fig 42), exhibited a reduction in the concentration per cell of each of the nucleotides present; AMP, GMP, ADP, GDP, ATP and GTP. It is probable that the nucleotides were being utilised during the growth of the organism, and as there was no nutrient stress imposed upon the cells, there was no production of 'Magic Spot'.

**Figure 42:** Growth (E470nm) (o) of *Bacillus subtilis* NCIMB 12900 in carbon (2.56x10<sup>-2</sup>M) and nitrogen (1.87x10<sup>-3</sup>M) sufficient L&C CDM against (Δ) AMP; (□) GMP; (▲) ADP; (■) GDP; (×) ATP; (+) GTP; concentration per cell (M). For reasons of clarity error bars have been deleted from this figure.



*Determination of the Nucleotide Concentration per Cell of **Bacillus subtilis** NCIMB 12900 Culture in Carbon and Nitrogen Sufficient L&C CDM challenged with 80% MIC Erythromycin.*

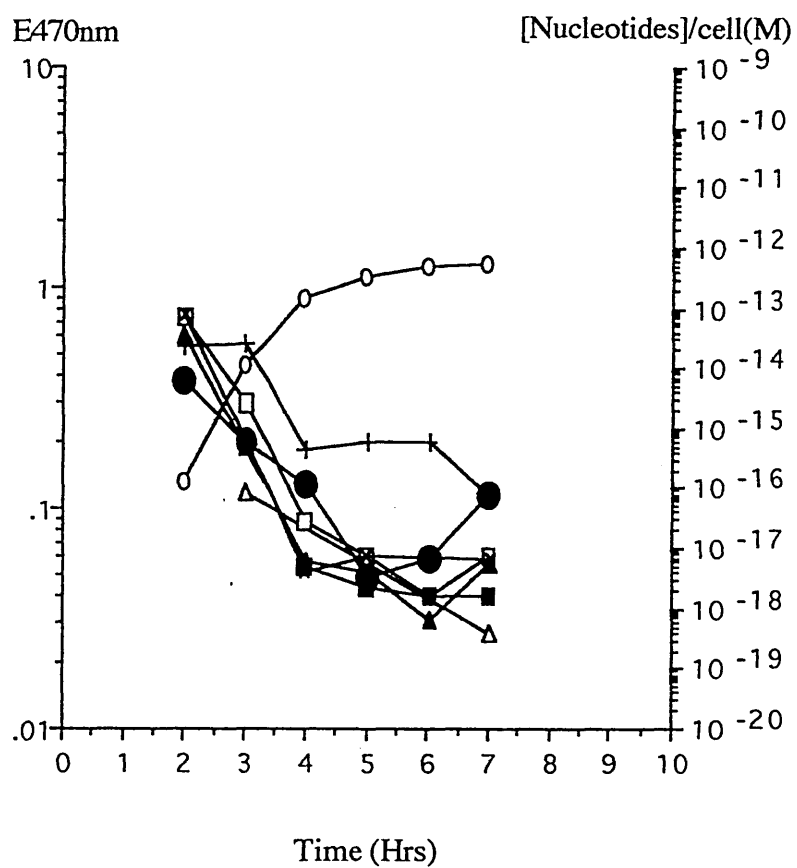
The carbon ( $2.56 \times 10^{-2} \text{M}$ ) and nitrogen ( $1.87 \times 10^{-3} \text{M}$ ) sufficient culture challenged with  $1.2 \mu\text{g mL}^{-1}$  erythromycin (Fig 43) exhibited a decrease in all the cellular nucleotides detected within the cell; AMP, GMP, ADP, GDP, ATP, and GTP. 'Magic Spot' was present after the addition erythromycin. It was observed to decrease in concentration per cell during exponential growth phase. As the cells entered stationary phase the concentration of 'Magic Spot' increased. The reduction in ATP and GTP was due to the general metabolic requirements of the cells and also to the production of pppGpp. The production of pppGpp was due to the presence of erythromycin as it was not produced in the control culture.

*Determination of the Nucleotide Concentration per Cell of **Bacillus subtilis** NCIMB 12900 Culture in Carbon Limited L&C CDM*

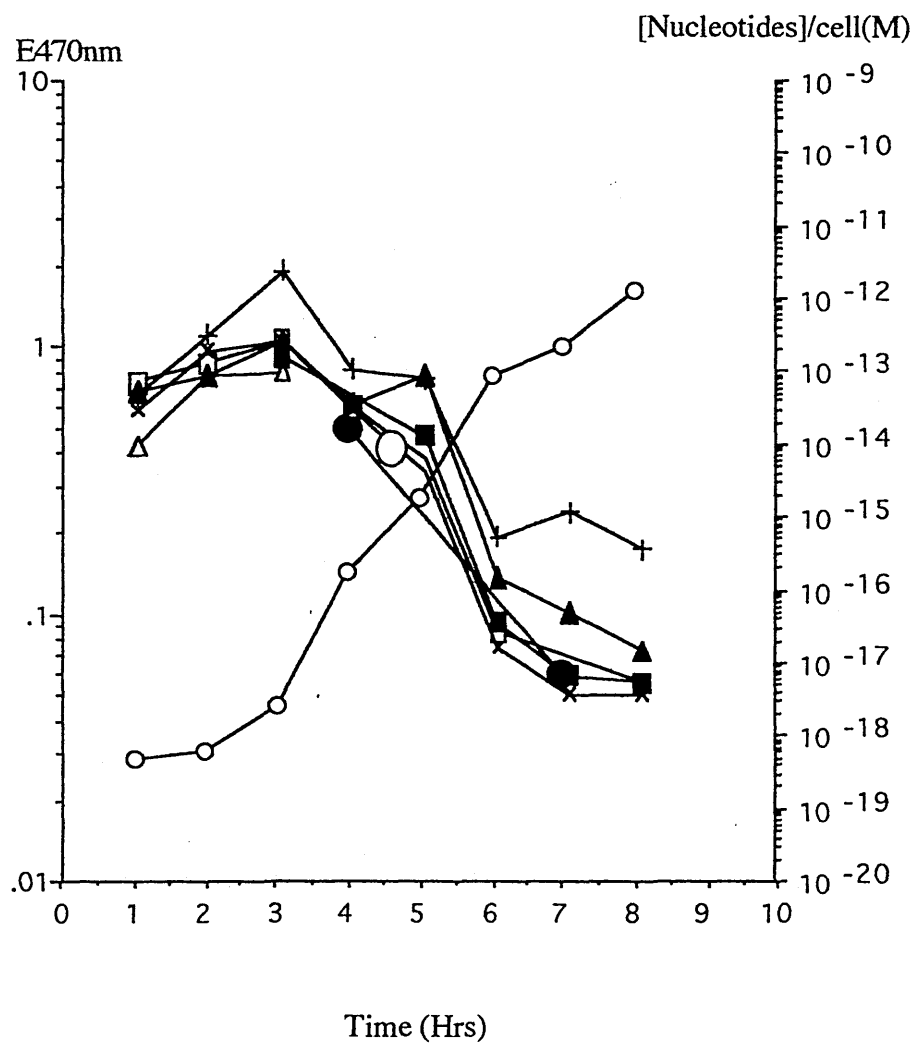
*B. subtilis* grown under carbon limited conditions ( $1.3 \times 10^{-4} \text{M}$ ) (Fig 44) exhibited an initial increase in the concentration per cell of the nucleotides AMP, GMP, ADP, GDP, ATP and GTP during lag phase. Once the cells began to grow exponentially these nucleotides began to decrease in concentration per cell, due to cellular metabolic activity, until the onset of stationary phase. The carbon limited culture of *B. subtilis* appeared to exhibit diauxic growth, due to the presence of two carbon sources within the medium (glucose and sodium citrate). During the second exponential growth phase the concentration of the nucleotides continued to decrease, but this was at a much slower rate. During the observation of exponential growth phase (2-6 hours), pppGpp was produced. This coincided with the reduction in concentration of both ATP and GTP. ppGpp was produced after 5 hours of incubation and coincided with the disappearance of GDP at 5 hours. The nucleotides continued to decrease until the growth rate of the culture began to slow down. pppGpp was detected once more at 7 hours which coincided with an observed reduction of GTP and ATP concentrations. Carbon limitation appears to be responsible for the induction of ppGpp and pppGpp.

**Figure 43:** Growth (E470nm) (o) of *Bacillus subtilis* NCIMB 12900 in carbon ( $2.56 \times 10^{-2} \text{M}$ ) and nitrogen ( $1.87 \times 10^{-3} \text{M}$ ) sufficient L&C CDM challenged with  $1.2 \mu\text{g mL}^{-1}$  erythromycin against ( $\Delta$ ) AMP; ( $\square$ ) GMP; ( $\blacktriangle$ ) ADP; ( $\blacksquare$ ) GDP; ( $\times$ ) ATP; (+) GTP; ( $\bullet$ ) pppGpp; concentration per cell (M). For reasons of clarity error bars have been deleted from this figure.





**Figure 44:** Growth (E470nm) (o) of *Bacillus subtilis* NCIMB 12900 in carbon limited ( $1.3 \times 10^{-4}$ M) L&C CDM against ( $\Delta$ ) AMP; ( $\square$ ) GMP; ( $\blacktriangle$ ) ADP; ( $\blacksquare$ ) GDP; ( $\times$ ) ATP; (+) GTP; (O) ppGpp; ( $\bullet$ ) pppGpp concentration per cell (M). For reasons of clarity error bars have been deleted from this figure.



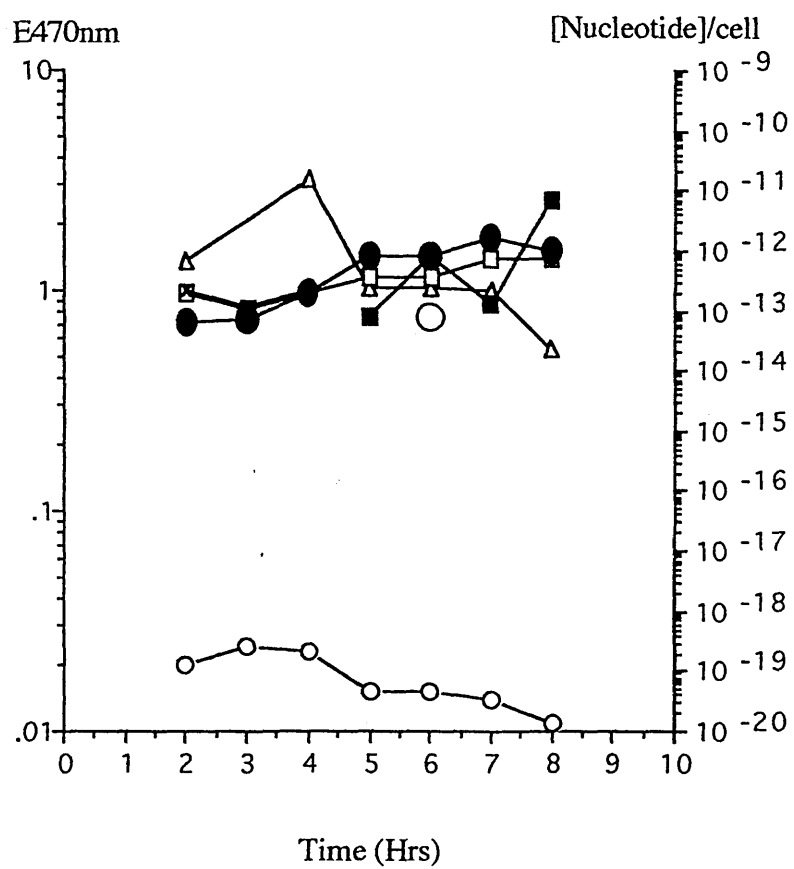
*Determination of the Nucleotide Concentration per Cell of **Bacillus subtilis** NCIMB 12900 Culture in Carbon Limited L&C CDM challenged with 80% MIC Erythromycin*

*B. subtilis* grown under carbon limited ( $1.3 \times 10^{-4} \text{M}$ ), conditions challenged with  $1.2 \mu\text{g mL}^{-1}$  erythromycin (Fig 45) exhibited no growth. ATP was only detectable from the hours of 2-4 after incubation. pppGpp was present throughout the growth of the culture and its concentration of was observed to increase gradually. This was accompanied by a decrease in ATP concentration. ATP was not detected after 4 hours and at this time the concentration of pppGpp increased. The concentration of pppGpp remained constant for 1 hour and at 6 hours ppGpp was detected, the constant concentration at this time was possibly due to the production of ppGpp. The concentration of pppGpp continued to increase as the cells density decreased. Erythromycin combined with carbon limitation inhibited the growth of the cells and also induced the production of 'Magic Spots' I and II.

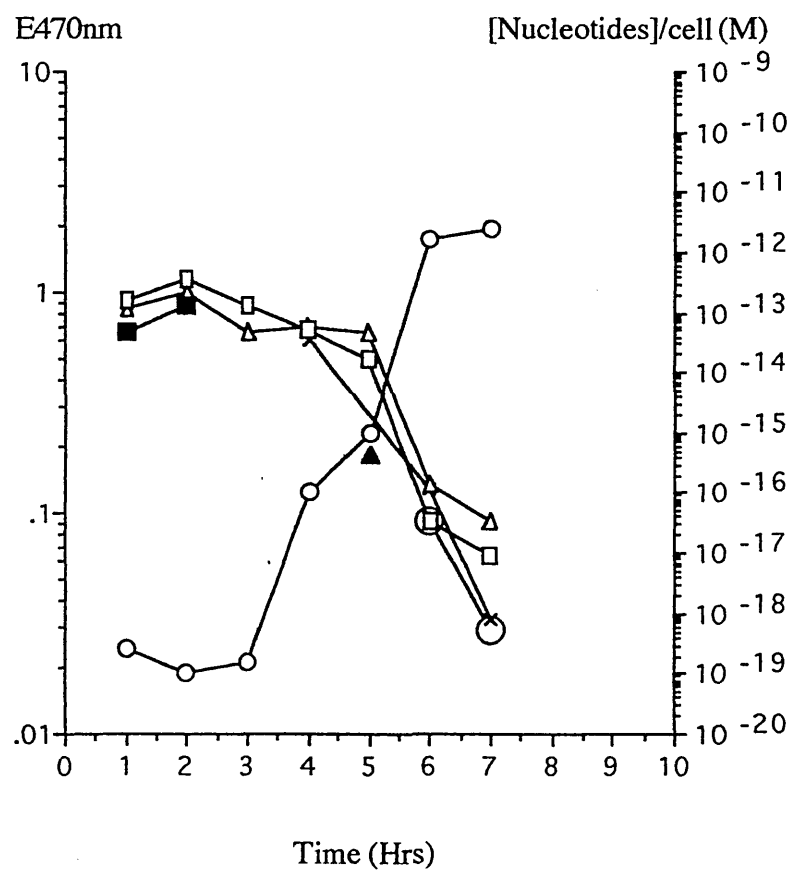
*Determination of the Nucleotide Concentration per Cell of **Bacillus subtilis** NCIMB 12900 Culture in Nitrogen Limited L&C CDM*

*B. subtilis* exposed to nitrogen limitation ( $4.8 \times 10^{-6} \text{M}$ ), (Fig 46) exhibited a reduction in the concentration per cell of nucleotides AMP and GMP throughout the growth of the culture. GDP was detected for the first 2 hours of analysis and ADP was detected after 5 hours for 1 hour only. ATP was detected from 4 hours and its concentration decreased until 7 hours (stationary phase). No GTP was detected, possibly due to its utilisation in the synthesis of protein. The HPN, ppGpp was detected at the onset of stationary phase after 6 hours of incubation and was still present after 7 hours. The production of 'Magic Spot' was due to the exhaustion of nitrogen in the L&C CDM. The detection of ADP at 5 hours may have been due to the cells initiating the production of 'Magic Spot'. The detection of ADP coincided with a decrease in ATP concentration and 1 hour later the detection of ppGpp. The liberation of ADP may be the resulting by-product from the enzymatic cleavage of a molecule of phosphate from ATP thus, resulting in a higher concentration of ADP in the cells.

**Figure 45:** Growth (E470nm) (o) of *Bacillus subtilis* NCIMB 12900 in carbon limited ( $1.3 \times 10^{-4} \text{M}$ ) L&C CDM challenged with  $1.2 \mu\text{gmL}^{-1}$  erythromycin against ( $\Delta$ ) AMP; ( $\square$ ) GMP; ( $\blacksquare$ ) GDP; ( $\times$ ) ATP; (O) ppGpp; ( $\bullet$ ) pppGpp concentration per cell (M). For reasons of clarity error bars have been deleted from this figure.



**Figure 46:** Growth (E470nm) (o) of *Bacillus subtilis* NCIMB 12900 in nitrogen limited ( $4.8 \times 10^{-6}$ M) L&C CDM against ( $\Delta$ ) AMP; ( $\square$ ) GMP; ( $\blacktriangle$ ) ADP; ( $\blacksquare$ ) GDP; ( $\times$ ) ATP; (O) ppGpp; concentration per cell (M)



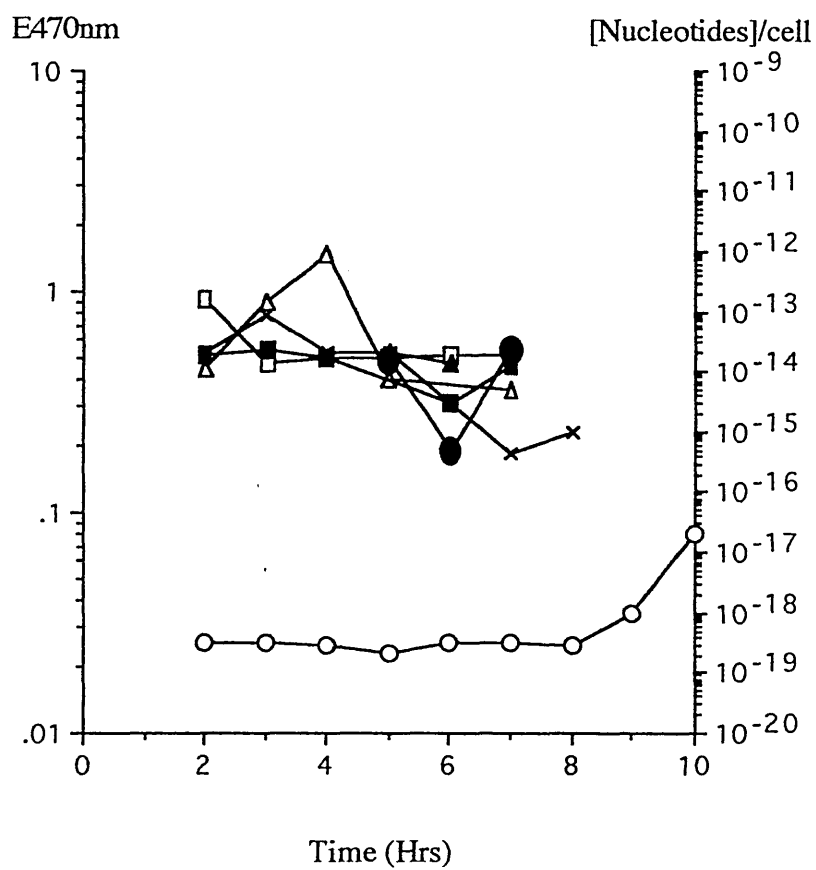


*Determination of the Nucleotide Concentration per Cell of **Bacillus subtilis** NCIMB 12900 Culture in Nitrogen Limited L&C CDM challenged with 80% MIC Erythromycin*

*B. subtilis* exposed to nitrogen limitation ( $4.8 \times 10^{-6} \text{M}$ ), challenged with

$1.2 \text{gmL}^{-1}$  erythromycin (Fig 47) exhibited no cell growth. This was due to the presence of erythromycin, as the nitrogen limited culture attained a higher cell density. There was very little reduction in the concentration per cell of GMP and GDP. ADP was detected after 5 hours of incubation and was present after 6 hours of incubation. It was not detected after 6 hours. The detection of ADP may be due to an increase in its concentration as a result of the enzymatic cleavage of phosphate from ATP during the production of 'Magic Spot'. AMP increased during the first three hours of incubation and may have been due to very little ATP being synthesised during that period, as there was no cell growth and the AMP was not being utilised in ATP biosynthesis within the cells. Once ATP was being utilised by the cells at 3-4 hours, the concentration of AMP decreased. The ATP concentration then remained constant for one hour, this was probably due to the fact that there was no cell metabolism or growth occurring. The concentration of ATP at 5 hours began to decrease. This reduction coincided with the production of pppGpp at 5, 6 and 7 hours. GDP was also detected at 5 hours, which coincided with the presence of 'Magic Spot'. The presence of erythromycin inhibited the growth of *B. subtilis*, and induced the production of 'Magic Spot'. There was no detectable GTP within the cells which could be due to the action of erythromycin which inhibits protein synthesis combined with the utilisation of GTP in the production of 'Magic Spot'.

**Figure 47:** Growth (E470nm) (o) of *Bacillus subtilis* NCIMB 12900 in nitrogen limited ( $4.8 \times 10^{-6}$ M) L&C CDM challenged with erythromycin against ( $\Delta$ ) AMP; ( $\square$ ) GMP; ( $\blacktriangle$ ) ADP; ( $\blacksquare$ ) GDP; ( $\times$ ) ATP; ( $\bullet$ ) pppGpp; concentration per cell. For reasons of clarity error bars have been deleted from this figure.



## **Production of 'Magic Spot' in *Escherichia coli* NCIMB 10000 and *Bacillus subtilis* NCIMB 12900**

The production of 'Magic Spot' in *E. coli* was not always at the same point of growth or necessarily under the influence of the presence of erythromycin. *E. coli* grown under carbon and nitrogen sufficient M9 medium challenged with erythromycin, produced 'Magic Spot'. These conditions produced both ppGpp and pppGpp. ppGpp was produced one hour after the addition of erythromycin and was present during the exponential phase. Once the cells entered stationary phase, pppGpp was produced and was detected at the 6 hour point.

Under conditions of carbon limitation the cells produced pppGpp during the exponential phase. Once the cells entered stationary phase there was no detection of this compound. The onset of stationary phase was very abrupt. This may have been due to the effects of carbon limitation combined with the utilisation of ATP in pppGpp production. Therefore, not enough ATP was available for normal cellular metabolism and division. The cells exposed to carbon limitation which were also challenged with erythromycin produced ppGpp after the cells had entered stationary phase, but this was for one hour only. It has been suggested that the immediate consequence of carbon limitation is the accumulation of ppGpp in stringent strains of *Vibrio* spp (Flardh *et al* 1994) and *E. coli* (Chaloner-Larsson & Yamazaki 1977, Cashel & Rudd 1987) and the consequence in *relA* strains was a fall in the [ATP]/[ADP] ratio. In both cases there was a simultaneous fall in GTP concentration (Flardh *et al* 1994, Flardh & Kjelleberg 1994). With *E. coli*, both accumulation of ppGpp and a reduction in both ATP and ADP were observed in carbon limited culture and carbon limited cultures challenged with erythromycin. This indicates that *E. coli* NCIMB 10000 produced ppGpp under carbon limited conditions and on exposure to erythromycin. Erythromycin induced the production of ppGpp, as it appeared only in the cultures which were erythromycin challenged. Starved *E. coli* cells undergo morphological and physiological adaptations (Bowman & Rand 1980). These adaptations to nutrient insufficiency include a reduction in their overall metabolic rate. The rapid reduction in protein synthesis, early onset of stationary phase and degradation of RNA suggests that these cellular constituents meet the requirements in order to maintain low endogenous metabolic activity, thus ensuring the survival of the bacteria (Bowman & Rand 1980). The responses of *E. coli* to these stresses may be due to the culture used, not being a dormant strain thus, stringent response is not

always exhibited when exposed to these stresses. The antibacterial spectra of all the macrolide antibiotics are essentially similar and are effective against Gram-positive bacteria, and some Gram-negative bacteria. One of the other antibiotics which was more effective against Gram-negative bacteria groups may have produced 'Magic Spot' under the conditions exposed to the cultures.

The response of *B. subtilis* to nutrient limitation and exposure to erythromycin is very different than that of *E. coli*. The only culture which did not produce 'Magic Spot' was the carbon and nitrogen sufficient L&C CDM. This may be due to the culture not being under stress. The other cultures (ie; nutrient limited and nutrient limited challenged with sub-MIC erythromycin) all produced 'Magic Spot' at some point during their exposure to these stresses. Carbon limitation produced both pppGpp and ppGpp. The responses to carbon limitation in *E. coli* resulted in the inhibition of stable RNA synthesis and the initiation of ppGpp and pppGpp production (Fehr & Richter 1981(a), Groat & Matin 1986, Cashel & Rudd 1987). The appearance of ppGpp in carbon limited cultures of *B. subtilis* is similar to responses exhibited in cultures of *E. coli* where only ppGpp was present and not pppGpp. It may also be the result of the degradation of pppGpp to ppGpp due to the enzyme pppGpp-5'-phosphohydrolase (Hara & Sy 1983). *B. subtilis* carbon limited cultures and carbon limited cultures challenged with erythromycin were the only ones which produced both HPNs. The presence of erythromycin initiated the production of pppGpp throughout the growth of the culture. Nitrogen limited cultures induced production of ppGpp only, whereas nitrogen limited cultures challenged with erythromycin produced pppGpp only. The results suggest that in *B. subtilis*, the presence of pppGpp under combined nutrient limitation and erythromycin challenge could be utilised as a marker for bacterial resistance to that antibiotic. The species of *Bacillus* used in the experiments was an endospore-forming strain. It is possible that this strain began to form spores as soon as the medium was inoculated, due to the nutrient limitation conditions and the combined nutrient limitation and antibiotic challenge. The ability of *Bacillus* to produce spores ensures their survival under extreme conditions and has an enormous influence upon the production of 'Magic Spot' in these cells. 'Magic Spot' was produced during exponential growth phase in cultures which were challenged with erythromycin. The cultures which were nutrient limited alone, 'Magic Spot' was produced towards the end of exponential growth phase or at the onset of stationary phase.

## DISCUSSION

Populations of bacteria which exist in a natural environment are exposed to a variety of environmental stresses. In order to survive hostile milieu, bacteria undergo severe adaptations. Nutrient limitation is the most common stress encountered by bacteria. Exposure to nutrient limitation results in the induction of either dormancy, stringent response or endospore formation. The production of the stringent response signal molecules, guanosine-3'-diphosphate-5'-diphosphate (ppGpp; MS I) and guanosine-3'-diphosphate-5'-triphosphate (pppGpp; MS II), is an example of one of the biochemical responses which occurs (Murao *et al* 1980, Pun & Pennington 1980, Nishino & Murao 1981, Mukai 1992). ppGpp and pppGpp are produced when bacteria are exposed to conditions of specific nutrient limitation, and nutrient limitation combined with exposure to antibiotics. These signal molecules appear either during exponential phase of growth (Eichel *et al* 1998), or as the culture enters stationary phase and sometimes during both phases. During stationary phase, bacteria produce many secondary metabolites. Included in these secondary metabolites is the production of antibiotics by Gram-positive bacteria. Antibiotic production is thought to enhance the survival of the bacteria existing in adverse environments (Katz & Demain 1977, Bennett & Bentley 1989, Vinning 1990). The production of ppGpp and pppGpp during the growth of bacteria, in particular stationary phase, and the subsequent production of secondary metabolites may indicate a link between the production of these novel compounds and bacterial resistance to antibiotics. The development of bacterial resistance and appearance of ppGpp and pppGpp, both occur under conditions of nutrient limitation and a combination of antibiotic challenged nutrient limited cells and therefore, may be linked.

In order to clarify a link between bacterial resistance and the production of the highly phosphorylated nucleotides (HPN's) ppGpp and pppGpp under conditions of nutrient limitation and nutrient limitation combined with antibiotic challenge, the responses of bacteria to specific nutrient limitation were investigated. Once the nutritional requirements of the bacteria were established the bacteria would then be exposed to a combination of nutrient limitation and antibiotic challenge. The experimental approach undertaken was to observe the effects of bacterial growth exposed to specific nutrient limitations, the extraction of cellular nucleotides and the isolation and identification of HPN's produced under these conditions.

## Experimental Approach

### • *Nutrient Limitation*

Nutrient limitation studies using carbon, nitrogen, magnesium and phosphorus limitation were performed in order to establish the effect they had upon the growth rate of the bacteria. From the observed results, the bacterial requirement ( $K_S$  value) for that specific nutrient was determined (Table 12). Thus, enabling the observation of the responses exhibited in cultures of *E. coli* and *B. subtilis* when exposed to both specific nutrient limitation and antimicrobial agents.

### • *Isolation and Identification of Nucleotides*

In order to determine the presence of nucleotides and HPN's in cultures of *E. coli* and *B. subtilis*, overnight cultures were collected and the cellular nucleotides extracted (Baracchini *et al* 1988; Little & Bremer 1982). The cellular nucleotides were identified using Two Dimensional Thin Layer Chromatography (2D-TLC) (Randerath and Randerath 1964) and High Performance Liquid Chromatography (HPLC) (Ochi *et al* 1981, Little and Bremer 1982).

### • *Estimation of Minimal Inhibitory Concentrations (MIC's)*

It was necessary to establish the lowest concentration of the antibiotic required to inhibit microbial growth (Minimal Inhibitory Concentration or MIC). The method used to estimate the MIC of antibiotics in this study was the tube dilution method (Bloomfield 1991) (Table 10). This was repeated for the four antibiotics of choice in CDM. The MIC's for the four antibiotics were lower in CDM than in nutrient broth (Table 20). Thus, indicating that growth rate had an effect upon the susceptibility to antibiotics or that some ingredient of the CDM inhibited the activity of the antibiotics.

### • *Observation of ATP Concentration*

The utilisation of ATP in the production of 'Magic Spot' may be indicated *via* the observation of alterations in intracellular ATP concentrations as a function of the bacterial batch growth cycle (Luciferase/Luciferin assay) (Wishart 1984). The concentration of ATP was followed throughout the growth of the cultures exposed to conditions of nutrient sufficiency and nutrient limitation, challenged and unchallenged with sub-MIC

erythromycin.

- *Determination of Concentrations of Cellular Nucleotides and 'Magic Spot' using HPLC.*

In order to determine the role of 'Magic Spot' in the development of bacterial resistance to antibiotics, the concentration of cellular nucleotides and HPNs in cellular extracts from *E. coli* and *B. subtilis* were observed throughout the growth of the organisms in batch culture. The nucleotides were extracted from cells of *E. coli* and *B. subtilis* ( Little & Bremer 1982, Baracchini 1988) which were grown under conditions of nutrient sufficiency and nutrient limitation, challenged and unchallenged with sub-MIC erythromycin. These cellular extracts were observed *via* HPLC (Ochi *et al* 1981, Payne & Ames 1982 and a technique developed in this study).

### **Effects of Specific Nutrient Limitation upon the Growth Rate of *Escherichia coli* and *Bacillus subtilis*.**

There are many responses and adaptations undertaken by bacteria in order to survive conditions of nutrient limitation, depending on whether they are sporulating (differentiating) or non-sporulating (non-differentiating) bacteria. In order to ensure the survival of the bacteria, these responses to nutritional deprivation are similar (Freese 1989). Microbial differentiation occurs as the result of the limitation of an essential nutrient. Bacteria tend to be more resistant to antimicrobial agents when they exist in a hostile milieu. This is due to the extended growth rate of the bacteria as a result of nutrient limitation. Thus, they are exposed to antimicrobial agents for a longer period of time due to the antimicrobial agent having longer to penetrate the cell (Koch & Gross 1979). Klemperer *et al* (1979) quantitatively determined the nutritional requirements of *E. coli* W3110 cells with and without the RPI plasmid in batch culture. Cells containing the R plasmid conferred greater resistance to antibiotics under conditions of nutrient limitation than those without the R plasmid. The nutrient being limited was also an important factor in the resistance developed by the cells, as carbon limitation resulted in a higher level of antibiotic resistance than phosphate limitation. The adaptations by the cells to nutrient limitation may explain the development of resistance of bacteria to antibiotics (Klemperer *et al* 1980).

*E. coli* and *B. subtilis* were grown in CDM which contained decreasing concentrations of each nutrient (Tables 4 & 5). The growth of both organisms was observed



spectrophotometrically (E470nm) and the growth rate and cell densities attained were noted. The  $K_s$  value (Table 12) for each organism and nutrient was determined from these results. Bacterial cultures exposed to nutrient limitation exhibited a general reduction in growth rate and cell density, which was more apparent in *E. coli* than in *B. subtilis* (Figs 12-19; Table 11). The onset of stationary phase in nutrient limited cultures of *E. coli* was earlier than that observed in the control culture. Whereas, the onset of stationary phase in nutrient limited cultures of *B. subtilis* was slightly later than that in the control culture or occurred at the same time, with the exception of carbon limitation which was earlier. As nutrient limitation affected the onset of stationary phase in both organisms, and it is at this time that secondary metabolites are produced and HPN's appear, bacterial cultures were grown for nucleotide analysis. Both *E. coli* and *B. subtilis* were grown under nutrient sufficient conditions overnight, in order to maximise the concentration of 'Magic Spot'. The cells were collected and the nucleotides were extracted and analysed for the presence of 'Magic Spot' using 2D-TLC and HPLC.

#### **Isolation and Identification of 'Magic Spot' in Bacterial Cellular Extracts.**

The isolation and identification of cellular nucleotides extracted from *E. coli* and *B. subtilis* was performed using 2D-TLC and HPLC. The nucleotides were extracted from the overnight cultures (Baracchini *et al* 1988, Little & Bremer 1982). It was thought that as the cells had exhausted the nutrients in the medium that 'Magic Spot' would be present. Separation of nucleotides using 2D-TLC was performed using standard nucleotides in order to obtain the  $R_f$  values for each nucleotide (Table 7) (Randerath & Randerath 1964). The cellular extracts from both organisms were separated according to the protocols of Randerath and Randerath (1964). The separation identified the nucleotides AMP, GMP, ADP, GDP, ATP and GTP from the cellular extracts of *E. coli*, but there was no detectable ppGpp or pppGpp present within these cells. The cellular extracts of *B. subtilis* were separated and the nucleotides identified were ADP, GDP, ppGpp and pppGpp. The lack of a commercially available 'Magic Spot' control proved difficult in the positive identification of ppGpp and pppGpp. 2D-TLC indicated that ppGpp and pppGpp was being produced in the cells of *B. subtilis* but was not conclusive for *E. coli*. Due to the lack of availability of a control ppGpp and pppGpp and published  $R_f$  values for *E. coli* made it necessary to employ a rapid technique of isolation and identification. The work performed by Ochi *et al* (1981), Payne

and Ames (1982), Little and Bremer (1982) and Baracchini (1988) in the successful separation of ppGpp and pppGpp using ion-exchange HPLC indicated that this was the most appropriate technique to use for the isolation and identification procedure.

Standard nucleotides AMP, GMP, ADP, GDP, ATP and GTP were assayed using the methods of Ochi *et al* (1981) and Little and Bremer (1982) (Table 8). The  $R_t$  values obtained were used along with the published data of Ochi *et al* (1981) as support for the verification of results obtained using HPLC identification. Problems were encountered with the ion exchange HPLC technique, the more serious one being the persistent appearance of a large peak at 52 minutes during the analysis of *E. coli*. Initially, this peak was thought to be 'Magic Spot', as it corresponded to the published data of Ochi *et al* (1981). Further analysis of other bacterial and nucleotide standards revealed this peak in every sample analysed by this method. Many alternative methods were tried, in order to solve this problem. It was possible that 'Magic Spot' would be masked by this unknown peak. Protein analysis (Lowry *et al* 1951) indicated there was no protein present in the fraction collected which produced the peak at 52 minutes. The fraction was analysed for nucleotide content (Adams *et al* 1992) and this indicated the nucleotide content in the fraction was between 8.5 and 9.3%. A specialist cleaning and regenerating regime was performed after every bacterial sample was analysed, in order to remove the unbound nucleotides from the column (page 112). This did not solve the problem as the peak at 52 minutes continued to appear in the subsequent traces of the standard nucleotides. Alternative methods (Payne & Ames 1982) were tried, but the published methods did not fully disclose the gradient programme utilised in these procedures. Therefore the results obtained utilising their methods could not be subsequently supported. Personal communication with R. England (University of Central Lancashire), also working in a similar area revealed that they had encountered the same problem. They confirmed that the peak at 52 minutes was a shift in base line due to the system switching from a mixture of low and high ionic strength buffer to high ionic strength buffer alone.

The work performed by Ochi *et al* (1981) isolated 'Magic Spot' at 52 and 60 minutes. Using the gradient programme of Ochi *et al* (1981), ppGpp and pppGpp were isolated and identified when high ionic strength buffer alone was used as the eluent. Therefore, a new isocratic system was developed in these studies, utilising high ionic strength buffer. It was imperative that a 'Magic Spot' control was obtained in order to

support the observed results. However, no 'Magic Spot' control was available from a commercial source or any of the other research groups contacted. This lack of availability resulted in the necessity of synthesising a 'Magic Spot' control in our laboratory.

Murao and Nishino (1973) had isolated the enzyme, nucleotide pyrophosphotransferase which produced 'Magic Spot', from a soil bacterium, *Streptomyces adaphospholyticus* A-4668. The enzyme catalyses the synthesis of pppGpp and ppGpp by the pyrophosphotransfer of phosphate molecules from ATP to GTP and GDP (Murao & Nishino 1974). Nucleotide pyrophosphotransferase is present in the culture filtrate of *Streptomyces adaphospholyticus* (Murao & Nishino 1974). 'Magic Spot' was synthesised according to the methods of Hamagishi *et al* (1975) using the culture filtrate (Table 18, page 118). The presence of 'Magic Spot' was isolated and identified using FPLC (Hamagishi *et al* 1975, Horrocks, AJH. University of Abertay Dundee, Personal Communication). Once the 'Magic Spot' control had been produced, the new isocratic HPLC system was established. Using the newly developed method (page 151) standard nucleotides and the fractions which contained 'Magic Spot' which were collected from the FPLC separation were analysed, in order to obtain their  $R_t$  values (Table 23). The system was successful thus, enabling the analysis of nucleotide extracts of cultures of *E. coli* and *B. subtilis* exposed to nutrient sufficient and limited conditions, unchallenged and challenged with erythromycin.

Due to pressure on time, as the result of the problems encountered with the HPLC analysis, it was decided to perform the experiments with one antibiotic only (erythromycin) and under conditions of nutrient limitation for two nutrients (Carbon and Nitrogen). Erythromycin's site of action is within the ribosome, and as HPNs are ultimately associated with the ribosome, the use of this antibiotic may indicate if there is a link between nutrient limitation, with and without antibiotic challenge with erythromycin, and the production of 'Magic Spot'. Carbon is required for growth and is an essential nutrient for normal cellular metabolism. Various workers (Fehr & Richter 1981a, Groat & Matin 1986, Cashel & Rudd 1987) all suggested that carbon starvation resulted in the initiation of ppGpp and pppGpp in *E. coli* and *Salmonella typhimurium*. Nitrogen was chosen as it is required for protein synthesis and its depletion initiates the sporulation of the Gram-positive bacterium *Bacillus subtilis* (Driks & Losick 1991, Dubnau 1993, Hoch 1993a, Ireton *et al* 1993).

## Bacterial Responses to Nutrient Limitation and Antibiotic Challenge

Both *E. coli* and *B. subtilis* were grown under conditions of carbon and nitrogen sufficiency challenged and unchallenged with sub-MIC erythromycin, carbon limitation and nitrogen limitation challenged and unchallenged with sub-MIC erythromycin (Tables 20, 21 & 22). The effects of carbon limitation and nitrogen limitation challenged with erythromycin upon cultures of *E. coli* and *B. subtilis* were observed. The onset of stationary phase of cultures grown in medium with a single nutrient as the limiting factor coincided with the production of the ppGpp and pppGpp. This may suggest a direct link between these compounds and the stationary phase phenomena exhibited by these cells. It is apparent that the stresses imposed upon the cells, under conditions of either nitrogen or carbon limitation combined with the presence of erythromycin, result in the induction of 'Magic Spot' synthesis.

Dinning (1995) suggested that ATP concentration in cells of *Pseudomonas aeruginosa* during stationary phase decreased rapidly. This could be directly linked to the production of 'Magic Spot' as it is produced at the onset of stationary phase and during stationary phase. The effects of these growth conditions were observed and quantified using Luciferin/Luciferase assay (Wishart 1984) by observing the ATP concentration per cell during the batch cultures of cells until the cultures entered stationary phase, (Figs 26-37). The concentration of cellular nucleotides was also observed and quantified using a newly developed isocratic HPLC method until the cultures entered stationary phase (Fig 38-47).

### *Observation of ATP Concentration.*

The observation of ATP content in the cellular extracts of *E. coli* during growth under carbon and nitrogen sufficiency (Fig 27) and both carbon (Fig 29) and nitrogen limited (Fig 30) conditions, challenged with sub-MIC erythromycin, exhibited a greater reduction in ATP concentration per cell than that observed in the cellular extracts of the cells grown under conditions of carbon (Fig 28) or nitrogen (Fig 31) limitation alone. The presence of erythromycin did not affect the overall growth of the bacteria under these conditions. However, nitrogen limitation reduced the growth rate of *E. coli* and thus, a very small reduction in ATP concentration was observed. *E. coli* are capable of scavenging nitrogen from other trace chemical constituents within their environment and it is possible that the observed growth was due to this ability. Conditions of nitrogen limitation challenged

with erythromycin, inhibited the growth of *E. coli* and consequently the concentration of ATP remained relatively constant ( $4.545 \times 10^{-16} \text{M}$  to  $3.049 \times 10^{-16} \text{M}$ ).

In cultures of *B. subtilis*, the presence of sub-MIC erythromycin in carbon limited medium (Fig 35) inhibited the growth of the cells and thus, there was no observed reduction in ATP concentration. The presence of sub-MIC erythromycin in cultures which were limited for nitrogen (Fig 37) resulted in a reduction in the attained growth of the cells and no marked reduction in ATP concentration. The observed ATP content in the control cultures (Fig 32), carbon (Fig 34) and nitrogen (Fig 36) limited cultures fell throughout the period of the experiment. The observed reduction in ATP concentration within the cells of both *E. coli* and *B. subtilis* was possibly due to normal cellular metabolic activity and also its involvement in the production of 'Magic Spot'.

#### *Detection and Quantification of 'Magic Spot' using a New Isocratic HPLC System.*

Using the newly developed isocratic HPLC system the cellular extracts from *E. coli* and *B. subtilis*, grown under conditions of nutrient limitation challenged and unchallenged with sub-MIC erythromycin, were assayed for the presence of nucleotides and 'Magic Spot'. No 'Magic Spot' was detected in cellular extracts from carbon and nitrogen sufficient cultures (Fig 40) and nitrogen limitation and nitrogen limitation challenged with sub-MIC erythromycin cultures of *E. coli* (data not shown). The HPNs, ppGpp and pppGpp, were detected in cellular extracts of *E. coli* grown under conditions of carbon and nitrogen sufficiency challenged with sub-MIC erythromycin (Fig 39). The production of 'Magic Spot' coincided with a reduction in the concentration of GMP, ADP and ATP. In cultures which were limited for carbon alone (Fig 40), pppGpp was detected. This coincided with a reduction in the concentrations of GMP, ADP and ATP. pppGpp was detected in cellular extracts from cells grown under conditions of carbon limitation challenged with sub-MIC erythromycin (Fig 41). There was an observed reduction in concentration of AMP, GMP, ADP and ATP in these cultures.

The HPN, pppGpp, was detected in the cellular extracts of *B. subtilis* grown under conditions of carbon and nitrogen sufficiency challenged with sub-MIC erythromycin (Fig 43). The production of pppGpp coincided with a reduction in AMP, GMP, ADP, GDP, ATP and GTP concentrations. Both ppGpp and pppGpp were detected in cellular extracts from cultures grown under conditions of carbon limitation (Fig 44). The production of

HPN's was accompanied by a simultaneous reduction in AMP, GMP, ADP, GDP, ATP and GTP concentrations. In cultures grown under conditions of carbon limitation challenged with sub-MIC erythromycin (Fig 45) both ppGpp and pppGpp were detected. There was also an observed reduction in the concentrations of AMP, GMP, GDP and ATP in the same cultures. However, little growth was attained under these conditions, and the reduction in nucleotide concentrations was relatively small. ppGpp was detected in the cellular extracts from cultures grown under conditions of nitrogen limitation (Fig 46) and this was accompanied with a reduction in the concentration of AMP, GMP, ADP, GDP and ATP. The HPN pppGpp was detected in cellular extracts from cells grown under conditions of nitrogen limitation challenged with sub-MIC erythromycin (Fig 47) and this coincided with a small reduction in the concentration of the cellular nucleotides AMP, GMP, ADP, GDP and ATP. The results observed using the newly developed isocratic HPLC technique successfully identified the presence of both Magic Spots I and II, ppGpp and pppGpp respectively.

#### *'Magic Spot' and Nutrient Limitation.*

The growth rate of bacterial cultures in general, is slowed down in order to fully utilise the nutrients available (Brown *et al* 1990, Kjelleberg *et al* 1993). Many workers have shown that the cell envelopes of nutrient limited cells are altered as a direct result of that limitation (Hansen *et al* 1975, Williams 1988, Brown *et al* 1990, Holmquist & Kjelleberg 1993). The cell envelope is extremely adaptable in both structure and composition and continually interacts with its environment (Brown & Gilbert 1995). This flexibility confers a great advantage upon the bacteria in a changing environment. The adaptations which occur in the cell envelope components, when the environment is nutrient limited or the bacteria change their growth rate, include changes in phospholipid composition (Benms & Proulx 1974, Gunter *et al* 1975, Cozens & Brown 1981), fatty-acid composition (Minnikin *et al* 1971a, b), metal cation composition and species (Kenward *et al* 1979), envelope-associated proteins and enzymes (Turnowsky *et al* 1983), extracellular enzymes (Ombaka *et al* 1983), and polysaccharides (Dean *et al* 1977). Subsequently these adaptations may influence microbial susceptibility to chemical antimicrobial agents (Brown 1977, Brown *et al* 1979, Brown & Williams 1985, Brown *et al* 1990, Gilbert *et al* 1990) and antibiotics (Finch & Brown 1973, Boggis *et al* 1979, Turnowsky *et al* 1983). When nutrients are scarce a rapid

response is exhibited by these cells. This is the simultaneous shutdown of RNA, protein and peptidoglycan synthesis (Kjelleberg *et al* 1993). The cell utilises alternative substrates, by the modification of the cell composition, or the reduction in the synthesis of macromolecules containing these nutrients. The cell surface is adapted to ensure the cell is more competitive in the uptake of these scarce nutrients to the cytosol. Chronic starvation in general, results in a slower growth rate thus, achieving the maximum growth potential of the culture under given environmental conditions. Chronic starvation can also provoke the synthesis of inducible enzymes and transport systems in order to overcome the nutritional stress by way of shifting to more energy-productive catabolic pathways. Protein mistranslation can occur as a result of low growth rates and these mistranslations may occur more frequently during the growth of the bacterial cells under nutrient limited conditions (Gallant & Foley 1979). In order to prevent these errors the cells produce ppGpp which increases the proofreading capacity of protein synthesised during periods of reduced growth rates. It also regulates some important enzymes involved in various metabolic pathways, as well as controlling the transcription of specific genetic units during nutritional stress (Hara & Sy 1983). This stringent response is similar, in many ways, to the sporulation observed in differentiating bacteria.

In these studies, the responses to nutrient limitation did not always result in the production of 'Magic Spot' in *E. coli*. 'Magic Spot' was not always detected at the same point during growth or necessarily as a result of the presence of erythromycin. *E. coli* grown in carbon and nitrogen sufficient M9 medium challenged with erythromycin, produced both ppGpp and pppGpp. After the addition of erythromycin ppGpp was produced and was detected during exponential phase. Once the cells entered stationary phase, pppGpp was produced and was detected for 1 hour only. When cultures of *E. coli* and *S. typhimurium* were starved of carbon their responses were similar to that for amino acid starvation, ie: the inhibition of stable RNA production and the initiation of ppGpp and pppGpp production (Fehr & Richter 1981(a), Groat & Matin 1986, Cashel & Rudd 1987). Production of both ppGpp and pppGpp coincided with entry into stationary phase. Growth of the cultures with a single substrate as the limiting factor may suggest the involvement ppGpp and pppGpp with the stationary phase phenomena exhibited by the bacterial cells. These phenomena include dormancy, stringent response, endosporulation, development of competence, antibiotic production, chemotaxis and degradative enzyme production (Brown 1977, Matin

1979, Jensen *et al* 1993).

The cell densities attained in carbon limited cultures of *E. coli* were comparable to those attained in the control cultures. Carbon limitation in *E. coli* resulted in the cells growing rapidly during the first few hours of growth. During this period of rapid growth, the cultures produced pppGpp. After the cultures entered stationary phase there was no further detection of pppGpp. However, cultures exposed to carbon limitation combined with erythromycin challenge produced ppGpp during stationary phase, but for one hour only. Flardh *et al* (1994) suggested that carbon limitation resulted in the accumulation of ppGpp in stringent strains of *Vibrio* spp. and in *E. coli* and that the result of carbon limitation in *relA* strains was a fall in the [ATP]/[ADP] ratio (Chaloner-Larsson & Yamazaki 1978, Cashel & Rudd 1987). A simultaneous fall in GTP concentration was observed in both *Vibrio* spp. and *relA* strains of *E. coli* (Flardh *et al* 1994, Flardh & Kjelleberg 1994). The growth of *E. coli* in carbon limited medium challenged with erythromycin exhibited the accumulation of ppGpp which coincided with the reduction in both ATP and ADP concentrations. This indicates that *E. coli* NCIMB 10000 produces ppGpp under conditions of carbon limitation combined with erythromycin challenge. These results indicate that erythromycin induces the production of ppGpp, as it appeared only in the *E. coli* cultures which were erythromycin challenged.

Cells which are starved undergo morphological and physiological adaptations (Bowman & Rand 1980). The adaptation exhibited by bacterial cells on exposure to nutritional stresses is the reduction in their natural metabolic activities. Growth may not always be slow in unfavourable conditions, bacteria often respond by shutting down metabolism and forming endospores, or temporarily entering a quiescent state (Hoch 1997). It is critical for the cell to respond rapidly to the environmental fluctuations in order to survive. This may be the reason why *E. coli* exhibited such rapid growth under carbon limited conditions.

Growth under the conditions of nitrogen limitation and nitrogen limitation combined with erythromycin challenge drastically reduced the cell density of the cultures compared to the control cultures, and there was no 'Magic Spot' production under these conditions. The lack of 'Magic Spot' production in cells grown in nitrogen limiting conditions may be due to the production of non-culturable and abnormal cells. Hengge-Aronis (1993) suggested that nitrogen and phosphorous starvation caused the production of



non-culturable and abnormal cells and that these conditions of limitation failed to provoke stringency. In other organisms stringency is involved in the regulation of starvation-induced genes and optimises bacterial survival in an environment under starvation stressed conditions. All the available nitrogen may have been utilised in protein synthesis and not in the production of 'Magic spot'. *E. coli* can scavenge trace nitrogen from other chemical sources within the medium, therefore, acquiring sufficient nitrogen for protein synthesis to maintain the viability of the cells. In order to remain viable in a nutrient limited environment the cell must synthesize proteins efficiently in the absence of exogenous substrates (Albertson *et al* 1990). The undetectable GTP levels during the growth of *E. coli* may have been a result of GTP being utilised in both protein synthesis and simultaneous 'Magic Spot' production. Protein synthesis can occur due to the substitution of pppGpp for GTP in protein synthesis, but subsequently pppGpp cannot be substituted in translocation processes. The failure of translocation of pppGpp may be due to the ribosomes not being able to hydrolyse pppGpp to GTP due to the position of the ribosomal 3'-pyrophosphate. The presence of erythromycin appears to be responsible for inducing the production of both ppGpp and pppGpp, as these compounds were not detected in the control cultures. These compounds (Magic Spots I and II) accumulate during various stresses; ie: nutrient limitation and exposure to antibiotics and thus, indicate the onset of stringent response. A rapid response to these stresses is critical in order to ensure the survival of the cells. However, once the nutrients become available, the starved cells resume growth and cell division (Kjelleberg *et al* 1993). The progeny of the cells which survive periods of nutrient limitation whilst challenged with sub-MIC concentrations of antibiotic are resistant to that particular antibiotic. The resistant cells confer their resistance properties to their subsequent daughter cells.

Nutrient limitation and combined nutrient limitation with the presence of erythromycin resulted in very different responses in cultures of *B. subtilis* than in *E. coli*. The only culture which did not produce 'Magic Spot' was the culture grown under conditions of carbon and nitrogen sufficiency in L&C CDM. The other nutrient limited and combined nutrient limited and erythromycin challenged cultures all produced 'Magic Spot' at some point during their exposure to these stresses. Conditions of carbon and nitrogen sufficiency challenged with erythromycin resulted in a reduction in concentration of all cellular nucleotides detected within the cell. 'Magic Spot' was present after the addition

erythromycin. During exponential phase it decreased in concentration, but as the cells entered stationary phase the concentration of 'Magic spot' increased. It is possible that the reduction in ATP and GTP was due to the general metabolic requirements of the cells; ie: normal cellular metabolism and protein synthesis, and also to the production of pppGpp. The production of pppGpp was due to the presence of erythromycin as it was not produced in the control culture.

*B. subtilis* grown in carbon limited medium produced both pppGpp and ppGpp during exponential phase and during the onset of stationary phase. The main HPN produced was pppGpp, it was detected throughout the observed growth of the organism whereas, ppGpp was detected for one hour only. *B. subtilis* grown in carbon limited medium challenged with erythromycin produced both HPN's. The presence of erythromycin appeared to inhibit growth and initiate the production of pppGpp throughout the period of observation of the culture.

The growth of *B. subtilis* under conditions of nitrogen limitation was not affected when compared to the growth of control cultures. This may have been due to there being two sources of nitrogen available within the medium (NH<sub>4</sub>Cl and L-Tryptophan). The concentration of L-Tryptophan was varied, in this experiment, in order to observe nutrient limitation effects, as the concentration of NH<sub>4</sub>Cl was already at it's limiting value. Therefore, there may have been sufficient nitrogen available for continuing protein biosynthesis and cellular metabolism, irrespective of the concentration of L-Tryptophan. These conditions initiated the production of ppGpp at the onset of stationary phase, which was accompanied by a rapid reduction in ATP concentration.

The addition of erythromycin to nitrogen limited cultures inhibited cell growth the cultures exhibited an extremely long lag phase (8 hours). Some growth was attained towards the end of the observed time of the experiment, and there was a reduction in ATP concentration as this growth was initiated. pppGpp was detected during lag phase. The concentration of pppGpp initially decreased, coinciding with a small increase in cell density. The pppGpp concentration increased and was accompanied with a decrease in the concentrations of both ATP and GTP.

The species of *Bacillus* used in the experiments was an endospore-forming strain. This strain may have initiated sporulation in some of the cells immediately after the medium was inoculated, in response to conditions of nutrient limitation challenged with

erythromycin. Bacteria are capable of detecting very small changes in their environment and they rapidly alter their metabolism to ensure the survival of the cells and their genomes. This may explain why 'Magic Spot' was detected during exponential phase cells as well as stationary phase cells. The ability of *Bacillus subtilis* to produce spores ensures their survival under extreme conditions and has an enormous influence upon the production of 'Magic Spot' in these cells. 'Magic Spot' was produced during exponential growth phase in cultures which were challenged with erythromycin. In the cultures which were nutrient limited alone, 'Magic Spot' was produced towards the end of exponential growth phase or at the onset of stationary phase. These results suggest that the appearance of pppGpp in *B. subtilis* exposed to conditions of nutrient limitation and combined nutrient limitation with erythromycin challenge could be utilised as a marker for bacterial resistance to that antibiotic. The growth of *E. coli* and *B. subtilis* under the conditions of nutrient limitation and nutrient limitation challenged with erythromycin induced the synthesis of 'Magic Spot'. The type of 'Magic Spot' produced appeared to be determined by the nutrient limited in the particular organism. The results suggested that *E. coli* induced ppGpp and *B. subtilis* induced pppGpp synthesis under specific stress conditions.

## Conclusions

'Magic Spot' was positively identified in cellular extracts of *B. subtilis* using 2D-TLC, but this was not the case with cellular extracts of *E. coli*. The use of gradient HPLC revealed the presence of Magic Spots in cellular extracts of both microorganisms, but the peaks were found to lie under and be obscured by the presence of a base line curve. Therefore, a new isocratic HPLC protocol was developed in these studies which identified and quantified the presence of 'Magic Spot' in both *E. coli* and *B. subtilis* cellular extracts. The Magic Spots were detected in the bacterial cells grown under conditions of specific nutrient stress with and without challenge with sub-MICs of erythromycin.

The synthesis of both ppGpp and pppGpp were induced in cultures of *E. coli* grown under conditions of carbon and nitrogen sufficiency challenged with sub-MICs of erythromycin. Carbon limitation only induced the production of pppGpp. Whereas, under conditions of carbon limitation challenged with sub-MIC erythromycin, ppGpp was produced. The detection of ppGpp in the cultures challenged with sub-MIC erythromycin, at either the onset of or during stationary phase of growth, suggests that this event may be

utilised as a marker for the development of bacterial resistance to antibiotics. This is because antibiotic challenged bacterial cultures will exhibit intrinsic antibiotic resistance at the onset of stationary phase and the two events are coincident.

The synthesis of ppGpp was induced in cultures of *B. subtilis* grown under conditions of carbon and nitrogen sufficiency challenged with sub-MIC erythromycin. However, conditions of carbon limitation and carbon limitation challenged with sub-MIC erythromycin induced the production of both ppGpp and pppGpp. Nitrogen limitation induced the synthesis of ppGpp and nitrogen limited conditions challenged with sub-MIC erythromycin induced the production of pppGpp. The detection of pppGpp in cultures of *Bacillus subtilis* NCIMB 12900 grown under conditions of nutrient sufficiency and nutrient limitation challenged with sub-MIC erythromycin, suggest that pppGpp production could be utilised as a marker for the onset of bacterial resistance to antibiotics.

## **SUGGESTIONS FOR FUTURE WORK**

- 1)**        The investigation of magnesium and phosphorous limitation in cultures with and without a sub-MIC challenge by either  $\beta$ -Lactam, 4-Quinolone or aminoglycoside antibiotics may reveal further evidence of the nature of this important marker system. This would require the refinement and adoption of the techniques exhibited within this study.
  
- 2)**        The utilisation of the radiolabelled phosphate bearing molecules both in 2D-TLC, HPLC and SDS-PAGE in order to further quantify and identify the production of the Magic Spots and other related compounds.
  
- 3)**        Analysis of bacterial cellular extracts using Nuclear Magnetic Resonance and Gas Mass Spectroscopy, in order to detect and quantify very low concentrations of 'Magic Spot' during all phases of the bacterial growth cycle.

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